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PATHWAY PROFILING OF REPLICATIVE AND INDUCED SENESCENCE

by

MAGGIE PURCELL

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2014

MAJOR: CANCER BIOLOGY

Approved by:

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Advisor   Date

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DEDICATION

To my wonderful husband and family.

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LIST OF ABBREVIATIONS

5-aza: 5-aza-2'-deoxycytidine

Adr: adriamycin

Nat: natural

Qui: quiescent

Imm: immortal

H_2O_2: hydrogen peroxide

LLP: lowest passage

LP: low passage

LFS: Li-Fraumeni Syndrome

IL1: interleukin 1

OIS: oncogene induced senescence

SAHF: senescence-associated heterochromatic foci

SASP: senescence-associated secretory phenotype

SA-b-gal: senescence-associated b-galactosidase

IRF: interferon regulatory factor

ROS: reactive oxygen species

PD: population doubling

RT-PCR: real time polymerase chain reaction

GGA: Genomatix Genome Analyzer

SPIA: Signaling Pathway Impact Analysis

IPA: Ingenuity Pathway Analysis

CHAPTER 1

INTRODUCTION

1.1 Summary

Senescence is a permanent withdrawal from cell cycle that occurs naturally at a cellular level in response to the shortening of telomeres. This shortening of telomeres is a result of the “end replication problem,” which occurs because the ends of the chromosomes cannot be effectively replicated. This natural “clock” serves to limit the number of cell divisions and therefore protects the cell from an extended lifespan and potentially carcinogenic mutations. However, senescence also occurs in response to external stresses to the cell, which is known as induced senescence. This study compares the mechanisms of natural senescence, a response to the shortening of telomeres during replication, with induced senescence by using a variety of drugs to induce senescence: 5-aza-2-deoxycytidine (a DNA demethylating agent), adriamycin (a chemotherapeutic drug), and H_2O_2 (an agent causing oxidative stress).

MDAH041 cells, which are fibroblasts isolated from a patient with Li Fraumeni Syndrome, have heterozygous alleles of p53 (one wild-type allele and one allele with a frameshift mutation causing a protein truncation). Fibroblasts from LFS patients can either undergo natural senescence with serial cell culture because of the wildtype, functional p53 or at a low frequency spontaneously immortalize once the wild-type copy of p53 is lost. Therefore, this cell model provides naturally senescent cells as well as immortal cells which can be treated with the aforementioned drugs resulting in induced senescence. Using these conditions, gene expression profiling was performed. Gene expression analysis revealed 48 genes differentially expressed specifically in all 4 senescence types compared

to the immortal control. Pathway analysis of these 48 genes from these 4 types of cellular senescence revealed several pathways, which are all involved in innate immunity, showing for the first time a common gene expression profile among different types of senescence, as well as a central role for the IFN pathway in both natural and induced senescence. Specifically, the IL1 pathway was found to be up-regulated in all 4 types of senescence compared to immortal proliferating cells.

1.2 Multistep Carcinogenesis

The development of cancer has long been considered to be a multistep mechanism, consisting of more than just one determining factor. Vogelstein's model of multistep carcinogenesis involved an average of 3 to 6 mutations during malignancy, either the loss of tumor suppressor genes or the activation of oncogenes [1]. Preceding Vogelstein, Farber described stepwise progression of cancer consisting of an initiation step, promotion and progression [2]. Initiation was shown to involve some mutagenic change in DNA, due to chemical interactions, radiation or other carcinogens. After initiation, promotion occurs, resulting in an expanded population of cells resembling the original initiated cell. Finally, progression results in a malignant transformation of cells which yields cancer [2].

Several studies showed that oncogenes alone could not transform normal cells. One example of this was the addition of an EJ oncogene (a mutant form of the human H-ras) to a normal hamster fibroblast line, which did not cause transformation. However, if this oncogene was added to the fibroblasts after being immortalized, malignant transformation was observed indicating several steps in the pathway to tumorigenesis [3]. Similarly, embryonic cells transfected with the Ras oncogene did not become transformed unless the cells were immortalized previously. If the Ras oncogene was introduced in conjunction with a Myc oncogene or the SV40 virus large T antigen, transformation did occur and the embryonic cells became malignant [4]. Therefore, tumorigenesis is multistep mechanism and is not the result of single genetic changes.

1.3 Cellular Senescence

In 1961, Leonard Hayflick observed the cellular changes that are now collectively known as cellular senescence [5]. Hayflick initially described the tendency for fibroblasts to replicate only a limited number of times, and then enter a growth arrest or replicative senescence after approximately 60-80 cell doublings [5]. This was later found to be due to the “end replication problem” resulting from inefficient replication of the chromosome ends during replication [8]. In addition to spontaneous senescence, cells can be induced to undergo senescence in response to a variety of stimuli such as DNA damage (as with chemotherapeutic agents like adriamycin), oncogenic/mitogenic signals (as with H-Ras activation in the cell and termed specifically oncogene-induced senescence or OIS) and cellular stress (such as the oxidative stress following H_2O_2 treatment) [5]. Cellular senescence represents a checkpoint applied to the cell cycle that prevents cells from accumulating mutations that could enable them to develop an indefinite lifespan or “immortality”, a step on the pathway to tumorigenesis.

The cells used in this thesis research are from patients with Li Fraumeni Syndrome, and provide a good example of the multistep nature of carcinogenesis. The cells contain heterozygous copies of p53 (one wild-type allele and one allele with a mutation), and therefore become immortalized upon the loss of the wild-type copy of p53. However, the loss of p53 was found to be necessary but not sufficient to cause immortalization, again indicating multiple steps in cancer development [6]. Additionally, these immortal cells are not yet transformed and require even further mutations to yield a cancerous phenotype. These mutations may be due to the genomic instability caused by p53 insufficiency. Upon transfection of a Ras-oncogene these immortal cells can become tumorigenic [7].

Telomere shortening leads to spontaneously senescent cells with a finite number of population doublings. The repetitive DNA segments at the ends of the chromosomes average approximately 12 kb in human cells, but a limited number cannot be synthesized at each round of replication, due to the "end replication problem." Therefore telomeres become shorter with age [8]. When the telomeres become too short, a DNA damage response is triggered through the activation of p53 and pRb [8]. Immortal cells must be able to overcome the shortening of telomeres in order to replicate indefinitely. The majority of cancers do this by expressing an enzyme called telomerase which adds the repetitive DNA sequences on to the ends of chromosomes so that the telomeres never become so short that they trigger a DNA damage response [9]. A less common method for cancer cells to maintain their telomere length is known as alternative lengthening of telomeres, or ALT. This mechanism employs homologous recombination to ensure the telomeres do not become too short and occurs in only 10-15% of cancers [10]. Appropriately, gene expression changes between young cells and naturally senescent cells are decreased when hTERT is expressed in the cells, indicating that most gene expression changes are indeed due to telomere shortening [11]. It is important to note for this study that human fibroblasts that contain telomerase and are immortal can still be induced to senescence with various chemical agents that induce DNA damage independent of the telomeric DNA shortening [12].

In addition to the bypass of senescence through activation of telomerase or ALT, it can also be bypassed by inactivation of tumor suppressors. For example, viral oncoproteins can bind and inactivate tumor suppressor genes. SV40 (simian virus 40) large T antigen can bind and inactivate the p53 and pRb tumor suppressors which causes a bypass in the

senescent response [13, 14]. The E6 protein of the human papilloma virus can bind and inactivate p53 which also yields a bypass of the senescent response [15]. Additionally, the oncoprotein E7 from the human papilloma virus can complex with and inactivate pRb [16, 17].

1.4 Known Senescence Genes

The two main mechanisms associated with cellular senescence involve the p53 and Rb pathways mentioned previously. Both proteins serve as tumor suppressors and protect the cell from acquiring genetic mutations or DNA damage by activating a cell cycle checkpoint, which prevents a cell from becoming malignant. In the event of telomere attrition, cytotoxic drugs, and oncogene introduction, ATM/ATR or CHK1/2 genes become activated, which leads to the accumulation of p53. Additionally, p53 can be activated by p14ARF, which binds to MDM2 (a ubiquitin ligase) and therefore prevents the degradation of p53 [18]. The accumulation of p53 leads to the subsequent transcriptional activation of cell cycle inhibitor p21cip1, and the activation of cellular senescence through the inhibition of cyclin/CDK complexes.

Alternatively, the Rb/p16 pathway can also lead to cellular senescence. $p16^{INK4a}$ has been shown to be up-regulated in stressed and senescent cells, thus inhibiting cyclin D and cyclin-dependent kinases. This prevents the phosphorylation of Rb, which leaves Rb available to bind E2F family members. The subsequent association of Rb and E2F renders E2F unable to bind target genes and activate them during S-phase of the cell cycle [18]. Therefore, up-regulation of p16 ultimately leads to transcriptional repression of cell cycle genes through E2F. This prevents the progression through cell cycle and leads to a phenomenon known as Senescence Associated Heterochromatic Foci, or SAHF, which is a condensed chromatin structure that further prevents E2F activity on target gene promoters [19, 20]. However, it has also shown that formation of SAHF may be cell-type and damage-type specific [20]. Rb1 is a member of the pocket protein family, consisting of two additional members p107 and p130, however inactivation of these two family members

are rarely observed in cancer and there is little evidence to show a role for them during senescence [18]. The INK4A-ARF locus which encodes both the p16 and p14 proteins is the most commonly mutated or deleted locus in human tumors, allowing the cell to bypass senescence by p53 and Rb simultaneously [18].

Whether senescence is induced by activation of p53 or p16, it is dependent on the type of stress inflicted upon the cell. However, in many cases, expression of both p53 and p16 remain after sustained senescence. For example, a senescence induced by the p53 response to DNA damage can also exhibit p16 activation after prolonged senescence due to p38-MAPK pathway activation and ROS production [21].

In addition to the tumor suppressor genes that are frequently altered in expression after senescence, there are many other genes that play a role in senescence, including genes that were identified in the Tainsky laboratory [22]. RNA expression was analyzed during senescence using fibroblasts from a patient with Li Fraumeni Syndrome, which is a heritable cancer syndrome caused by a germline mutation in one allele of p53 [23]. The heterozygous mutation in p53 creates a unique cellular model system in that the fibroblasts can either enter senescence with telomere attrition (because of the wild-type allele of p53), or can undergo loss of the wild-type copy of p53 which leads to spontaneous immortalization. This characteristic is not observed in any other genetic syndrome [24]. These cells were previously used in our lab to study immortalization and senescence induced by treatment with 5-aza-2'-deoxycytidine, showing that 5-aza reverses epigenetic silencing of genes during immortalization. That study used Affymetrix microarray analysis to identify genes with altered expression in senescence. By comparing immortal LFS fibroblasts with senescent LFS fibroblasts induced into senescence by treatment with 5-

aza, it was found that there are 3 critical pathways involved in cellular immortalization: cytoskeleton, cell cycle and interferon pathways [22]. Cytoskeletal changes were not surprising, given the large flattened morphology of fibroblasts during senescence. Cell cycle gene expression changes were also not surprising, because the cells must withdraw from the cell cycle in order to cease division and enter senescence. However, the interferon pathway's involvement in senescence was an unexpected finding of the study. Additionally, senescent-associated genes were identified by identifying the unique subset of genes that were both up-regulated in senescence and down-regulated in immortalization, in all 4 of the LFS cell lines used. This approach also identified a list of 14 genes that met these criteria: ALDH1A3, CLTB, CREG, CYP1B1, FLJ14675, HPS5, HSPA2, HTATIP2, IGFBPrP1, KIAA1750, MAP1LC3B, OPTN, SERPINB2 and TNFAIP2 [22]. When identifying possible senescence genes, one strategy is to look at genes that are up-regulated in senescence and down-regulated in immortalization because senescence has been shown to be a dominant pathway [25].

1.5 Senescence as a Tumor Suppressive Mechanism

Senescence provides a barrier to tumor progression both in vitro and in vivo, as evidenced through several studies, including a study of the E2F3 transcription factor, in which E2F3 was ectopically expressed in the pituitary gland, leading to hyperplasia, but subsequently led to induction of senescence [26]. Additional studies include the discovery of senescent cells in benign naevi (moles) which typically exhibit BRAF oncogenic mutations and a study in which RasV12 was conditionally expressed in a mouse lung, leading to pre-neoplastic lung adenomas but rarely progressed to neoplastic adenomas [18, 27]. Senescence in the presence of an oncogene is termed oncogene-induced senescence or OIS. The role of senescence in preventing progression of pre-neoplastic lesions to malignant cancer has been shown in cancers of the prostate, colon, lymph and breast [18]. Benign melanocytic nevi in vivo were found to contain BRAF mutations, concurrent with positive staining for senescence-induced β-galactosidase staining [28]. This would indicate that when a cell obtains an oncogene, a form of senescence is activated to prevent proliferation of that cell. Additionally, markers of senescence have been found in vivo in several types of early neoplastic tissues, such as lung adenoma, mammary tumors, lymphomas, liver carcinomas and prostate neoplasias [29]. Therefore, evidence indicates that senescence is a barrier to tumorigenesis. The senescence that occurs as a result of activation of an oncogene such as Ras or BRAF is a known natural cellular mechanism of carcinogenesis suppression. It is thought that the increased replication due to oncogenic activation causes an abundance of DNA damage and consequently a DNA damage response via the p53 and pRb pathways [30]. It has also been proposed that the DNA damage response can be triggered by increased levels of ROS, which is a consequence of

oncogenic Ras expression [31]. Oncogene-induced senescence is a recently-identified mode of tumor suppression, similar to the classic tumor suppression mechanism of cell cycle arrest. However, oncogene-induced senescence is thought to be irreversible, whereas generic cell cycle arrest (quiescence) due to DNA damage can be reversed if the DNA damage is repaired and the cell is allowed to re-enter the cell cycle [32]. In addition to oncogene-induced senescence and cell cycle arrest, tumor suppression largely occurs via apoptosis when damage to the cell is too extreme to repair [32]. The tumor suppressor p53 dictates whether a cell will undergo senescence, quiescence or apoptosis in response to DNA damage [33]. Under normal conditions, p53 has a low level of expression, which is maintained through MDM2, an E3 ubiquitin ligase that ubiquitinates p53 and targets it for degradation [33].

1.6 Senescence Markers

Markers of senescence include prolonged withdrawal from cell cycle, enlarged morphology, senescence-associated β-galactosidase staining, increased levels of p16 and p21 and the formation of senescence-associated heterochromatic foci (SAHF) as well as a senescence-associated secretory phenotype (SASP). Senescence-associated β-galactosidase staining is an assay that results from the accumulation of β-galactosides within the lysosome of senescent cells due to enlarged lysosomal mass [34]. Because of this, β-galactosidase is able to catalyze the hydrolysis of β-galactosides into monosaccharides, which then results in the cleavage of 5-bromo-4-chloro-3-indolyl- β-D-galacto-pyranoside (X-gal), which leads to a blue precipitate at pH 6.0 [35]. Another indicator of senescence is the decrease in expression of cell-cycle and proliferation related genes, such as cyclin A and cyclin B, though these reductions in expression are not specific to senescence but rather the withdrawal of the cell from the cell cycle [36]. Additionally, the increase in expression of cyclin-dependent kinase inhibitors such as p21 can also indicate senescence [37].

SAHF or senescence-associated heterochromatic foci are areas of transcriptionally silent and compacted chromatin that result from the presence of repressive histone mark H3K9me3 and absence of activating histone mark H3K4me3 [38]. It is probable that SAHF maintains the cell in a non-proliferating state, as the E2F transcription factors cannot bind to the compressed and therefore inaccessible chromatin, which prevents transcription of S-phase cell cycle related genes [19]. The occurrence of SAHF is dependent upon the cell type, as well as type of damage inflicted, but seems to be consistently associated with the activation of the tumor suppressor p16 [20]. In oncogene-induced senescence, the

formation of SAHF occurs concurrently with DNA damage response and also correlates to the increased expression of p16 [39].

The senescence-associated secretory phenotype is a collection of factors that are secreted from senescent cells [40]. The factors are considered to be inflammatory, including members from the IL1 family, proteases, MMPs and chemokines, several of which were identified in our RNA-seq to be up-regulated in senescence [41]. The effect of the SASP is controversial, with evidence for these factors causing paracrine senescence to surrounding cells or causing chronic inflammation and therefore contributing to malignant phenotypes [41]. Secreted factors are also thought to attract immune factors to aid in the elimination of senescent cells [42].

1.7 Senescence Induction

Senescence can be induced by a variety of mechanisms. Firstly, oncogene-induced senescence can be achieved through the expression of an oncogene such as a mutated Ras [39]. Secondly, there are several chemical agents that can induce senescence through DNA damage or other stresses, such as chemotherapeutic agents and DNA damaging agents [43]. Thirdly, senescence can be induced through the re-activation of tumor suppressive pathways such as p53 or p16 by re-introducing these genes back into cells or reversing their silencing through epigenetic remodeling [44]. Lastly, genes that are up-regulated during senescence such as IRF5 and IRF7 can be ectopically expressed in cells to cause senescence [45]. This project utilizes hydrogen peroxide, adriamycin and 5-aza-2-deoxycytidine to induce senescence.

H_2O_2 (hydrogen peroxide) has been shown to induce senescence in various cell types at sublethal concentrations [46]. At high concentrations, H_2O_2 treatment can result in apoptosis. H_2O_2-treated cells were shown to be arrested in G1, which reflects senescent arrest [46]. p53 is temporarily up-regulated in these cells, while p21 showed a long-term up-regulation in response to H_2O_2 exposure [46]. Rb maintains low levels of phosphorylation, which is consistent with the senescent response: when Rb is not phosphorylated, it binds E2F and prevents transcription of proliferation-related genes [46]. H_2O_2 treated cells have been found to be senescent-associated β-galactosidase positive and have irreversible growth arrest [47].

5-aza-2-deoxycytidine is a DNA methyltransferase inhibitor that results in demethylation of promoters within the genome. 5-aza is a cytosine analog that incorporates into DNA, and covalently traps DNMTs, preventing further methylation activity [48].

Because many CpG islands in tumor suppressive promoters are frequently hyper-methylated in cancers, the demethylating action of 5-aza-2-deoxycytidine causes senescence through reactivation of tumor suppressors epigenetically silenced during immortalization, such as p16 [49]. 5-aza treatment of immortal LFS cells (the same type of cells used in this study) has shown three pathways affected by treatment: cytoskeletal pathway, cell cycle pathway, and the interferon pathway [22]. Further study has shown the loss of expression of the interferon pathway in immortalization of these cells, which can be thought of as the inverse of senescence [22]. High levels of histone 3 lysine 9 (H3K9) methylation have been shown in heterochromatin silenced genes, but this effect is reversed in response to treatment with 5-aza, indicating a possible role of 5-aza in histone demethylation [50]. An additional study showed that treatment of cells with 5-azacytidine (a DNMT inhibitor that incorporates into both the DNA and RNA) caused changes in histone modification patterns [51].

Adriamycin is an anthracycline antibiotic that is frequently used in the treatment of breast cancer. This drug typically causes induction of apoptosis when used chronically, however short-term acute treatment favors the senescent pathway [52]. Treatment with adriamycin has been shown to cause an increase in p53 levels, and decrease in telomerase levels, though the mechanism of action seems to be telomere length independent [52].

1.8 Senescence and Aging

Aging at a cellular level occurs through the shortening of telomeres, which limits the number of times a cell can proliferate. When the telomeres become critically short, a DNA damage response is triggered and the cell enters senescence rendering it unable to replicate [53]. As an organism ages, telomere length of cells is known to decrease overall, and the number of senescent cells with critically short telomeres is known to increase [53]. Additionally, syndromes of premature aging exhibit shortened telomeres compared to normal cells of these patients [53]. Several aging-related diseases such as infertility and digestive tract atrophies have also been shown to have shortened telomeres [53]. Telomerase-deficient mice showed early aging but a higher resistance to cancer, and telomerase-null mice showed a decline in longevity [54].

Aging at an organismal level is a risk factor for a myriad of disorders, including stroke, heart disease, cancer, dementia, osteoporosis, kidney failure, blindness and arthritis [55]. Many of these disorders are a direct result of chronic inflammation, which has recently been found to be in part due to the senescence-associated secretory phenotype [55]. It has also been proposed that the elimination of senescent cells may reduce chronic inflammation and therefore decrease the amount of chronic disease due to aging [55]. Early hypotheses about senescence's role in aging came from knowledge that tumor suppressors such as p53 and p16, which are up-regulated during senescence, cause mortality due to cancer at a young age when ablated in mice. However, until recently it was difficult to prove the involvement of senescent cells in the aging phenotype due to lack of in vivo markers for senescence. The most common markers for in vivo detection of senescence are senescence-associated β-galactosidase and elevated levels of p16; however p21,

macroH2A (a histone variant), IL-6 and DNA damage can also be observed [21]. Using these markers, senescent cells have been shown in age-related diseases such as osteoarthritis, pulmonary fibrosis and Alzheimer's disease [21].

Further evidence for senescence in aging comes from a study of selective p16 elimination from a mouse model. Using a transgene INK-ATTAC (which targets p16 for destruction through caspase activation), cells showing a high level of p16 (senescent cells) were selected for apoptosis. This selective elimination of p16 halted the progression of aging disorders in skeletal muscle and fat [56]. The role senescent cells play in tissue degeneration and organ dysfunction is unknown. It is possible that degeneration and dysfunction are caused by a simple decrease in tissue regenerative potential, but several things are thought to contribute to this phenotype. The microenvironment of aged cells limits stem cell viability, and regenerative potential of stem cells is improved when introduced to a "young" cell microenvironment [21]. Similarly, senescent cells secrete proteases that can disrupt tissues and membrane-bound receptors, as well as other components of the environment [21]. Other secreted factors such as IL6 and IL8 can stimulate tissue fibrosis. As a whole, the senescence-associated secretory phenotype can cause chronic inflammation which is associated with aging and the consequent development of age-related diseases [21]. These inflammatory factors can "spread" senescence to surrounding cells which exacerbates the senescent phenotype and tissue degeneration, but these factors can also promote survival and proliferation, which is consistent with the notion that cancer drastically increases in old age [21]. This may explain why the production of senescent cells increases with time, or it is also possible that

the elimination of senescent cells simply decreases with time which would cause an accumulation of senescent cells.

1.9 Antagonistic Pleiotropy

Antagonistic pleiotropy as it pertains to senescence was introduced in 1957 by George Williams. Briefly, antagonistic pleiotropy describes a phenomenon in which organisms can evolve mechanisms that hinder their overall survival but they are beneficial early in life [57]. Williams asserted that natural selection will promote genes beneficial during youth at the expense of adult life, because “an advantage during the period of maximum reproductive probability would increase the total reproductive probability more than a proportionately similar disadvantage later on would decrease it” [57]. Senescence fits this definition of antagonistic pleiotropy because it is beneficial early in life, serving to limit cancers but as an organism ages senescence will cause frailty, decreased regenerative capability and a general “old age” phenotype.

There is some evidence against senescence as antagonistic pleiotropy. Although the senescence-associated secretory phenotype is generally thought to have a negative effect because the secreted factors can promote cancer, some believe that the secreted factors are beneficial as they promote clearance by the immune system [58]. Senescence also plays a role in wound healing and tissue repair, which can prevent organ degeneration and tissue fibrosis and is therefore beneficial in late life [58]. It has also been noted that there is no definitive evidence that cites senescence as being more beneficial in young life compared to late life, or conversely that the detriments of senescent are more prevalent in late life than young life [58].

1.10 Senescence and the Interferon Pathway

Previous analysis of senescence pathways was performed in the Tainsky laboratory through gene expression studies of immortal LFS cells, proliferating versus those induced into senescence by 5-aza-2'-deoxycytidine. Three main pathways were found to be altered in immortalization and senescence: cell cycle, cytoskeleton and the interferon pathway [22]. Cell cycle was expected to be altered, due to a senescent cell's halt in growth and associated withdrawal from cell cycle. Cytoskeletal alterations are also not surprising given the stretched and enlarged morphology of a senescent cell. Therefore, the surprising finding was that of the interferon pathway, and this was studied extensively.

The Tainsky laboratory previously identified several interferon genes epigenetically silenced during immortalization [22, 48]; these genes were also found to be up-regulated in both natural senescence and senescence induced by 5-aza-2'-deoxycytidine [49]. Further confirmation of these genes showed that overexpression of interferon regulatory factors IRF5 and IRF7 was able to inhibit growth and induce senescence in immortal LFS cells [45]. However, it was also shown that STAT1 expression was not sufficient to regulate this senescent interferon response [25]. Immortal cells with abrogated interferon signaling had a higher tolerance to miRNA created by overexpression of DICER, while cells with a normal interferon response responded to overabundance of miRNA with cell death, inhibition of growth and senescence. This indicates a role for abrogation of the interferon pathway in early immortalization [59]. Because these changes were shown only in 5-aza-induced senescence, which is essentially the reversal of immortalization-related epigenetic silencing through the removal of methylation marks, the current study focused

on other types of induced senescence to assess whether the interferon pathway played a role in other types of senescence with different mechanisms of induction.

Other laboratories have reported a role for the interferon pathway in cellular senescence. Senescence induced by 5-bromo-2'-deoxyuridine, distamycin, aphidicolin or hyrdoxyurea in both normal and cancer cells were found to activate JAK/STAT signaling, expression of interferon-stimulated genes such as the IRFs (interferon regulatory factors), as well as several interleukins and interferons [60]. Knockdown of JAK1 in this study was found to abrogate the expression of interferon-stimulated genes [60]. It has been shown that interferon cytokines are inhibitors of cell growth, both in vivo and in vitro, which is consistent with the idea that they are involved with senescence [61]. cDNA microarray analyses showed the interferon pathway to be silenced in a model of human prostate cancer, and up-regulated in senescent human prostate epithelial cells [62]. Analysis of prostate cancer cells via serial analysis of gene expression (SAGE) showed up-regulation of interferon genes [63]. The specific role of interferon genes in cellular senescence and immortalization is largely unknown, however a small number of specific interferon genes have been characterized, such as Choubey et al. who identified IFI16 as a key regulator of senescence in prostate epithelial cells [64]. Another example is IFN-γ which has an anti-proliferative effect on gastric cancer cells [65] and can induce senescence in normal melanocytes [66].

1.11 The Interleukin 1 Pathway and Senescence

The senescence associated secretory phenotype is known to include members of the Interleukin 1 (IL-1) pathway [41]. IL1-α or IL1-β bind to the IL1 receptor (IL1R) and cause interaction with Myd88, an adaptor protein [67]. Activation of this adapter then causes downstream activation of IRAKs (interleukin-1 receptor associated kinases) and consequent activation of NF-kB [67]. The activation of NF-kB is thought to trigger the release of inflammatory cytokines that comprise the senescence associated secretory phenotype, including IL1-α, IL1-β, IL-8 and ICAM1 among several others. IL1-α is mainly membrane-bound, while IL1-β is mainly secreted when activated [68].

IL1-α was found to be necessary for the secretion of downstream inflammatory factors during senescence, such as IL-6 and IL-8 [68]. During senescence, fibroblasts were found to have high amounts of membrane-bound IL1-α, intracellular IL1-α and IL1-α mRNA, however very little secreted IL1-α [68]. Neutralizing IL1-α levels through IL1-α antibodies, IL1-α RNA interference and an IL1R antagonist all caused a decrease in senescence-associated IL-6 and IL-8 secretion, however the effect on amount of senescence was not noted in that study [68]. This study also showed a decrease in paracrine effects of the inflammatory phenotype: conditioned media from senescent cells depleted in IL1-α did not have as much of an invasive phenotype in metastatic cancer cells [68].

Conditioned media from cells undergoing replicative senescence, oncogene-induced senescence or drug-induced senescence contains high levels of Il-1, IL-6 and TGFβ, which can cause increased DNA damage by ROS [69]. This media is able to induce DNA damage and senescence in bystander cells [69]. Briefly, young BJ fibroblasts were exposed to

senescence-conditioned media from BJ fibroblasts undergoing oncogene-induced senescence, replicative senescence, or drug-induced senescence. The young BJ cells exhibited increased levels of γH2AX (a marker for DNA damage) as well as increased levels of senescence-associated β-galactosidase staining indicating senescence [69]. Additionally, U2OS osteosarcoma cells with a stably expressed GFP tag were mixed with U2OS cells that had undergone drug-induced senescence, and the GFP-positive cells showed an increase in γH2AX DNA damage foci [69]. Similarly, independent studies showed that oncogene-induced senescence activates a senescence-associated secretory phenotype and media from these cells can cause paracrine senescence through the IL-1 signaling network [70]. However, this study found that while IL1-α alone can induce a senescence-associated secretory phenotype when up-regulated, neutralization of both IL1-α and Il1-β through antibodies or through the knockdown of the IL1R was necessary to block the senescence-associated secretory phenotype [70]. The increase in IL-1α is due to senescence-associated changes in steady-state H_2O_2 levels, and therefore intracellular Ca^{2+} levels which promote calpain activation and causes cleavage to mature IL1-α [71].

1.12 Senescence and Cancer Therapy

The controversy surrounding senescence's usefulness during cancer therapy greatly increased with the discovery of the senescence-associated secretory phenotype. Senescence was initially thought to be a beneficial outcome of cancer therapy, rendering the cells unable to replicate and therefore prevent tumor growth. However, it has also been shown that senescent cells secrete inflammatory factors that can lead to chronic inflammation and consequently tumorigenesis [40]. Another theory is that senescence improves cancer therapy, but the senescent cells need to be expediently removed to avoid negative consequences of senescence [72]. Other approaches include preventing senescence altogether, or targeting the SASP to eliminate negative repercussions of lingering senescent cells [55]. Chronic inflammation as a result of normal aging is the leading cause of dementias, depression, atherosclerosis, cancers, diabetes and mortality [55]. This chronic inflammation may be due to an accumulation of senescent cells as the body ages.

It is has been shown that senescent cells can be removed through the targeting of the biomarker p16. In a study with BubR1 progeroid mice as a model, a transgene INK-ATTAC was administered, and upon addition of a drug, the p16-positive cells, which are considered senescent, were removed through apoptosis. When the p16-positive cells were removed throughout the mouse's entire lifespan, age-related pathologies were prevented in tissues such as adipose, skeletal muscle and eye, where p16 expression is generally increased with age. Additionally, when the p16-positive cells were cleared in late-life mice, the age-related disorders halted progression [56]. That study not only indicates a causal role for senescence in aging-related pathologies, but provides a proof of principle

that it may be possible to eliminate senescent cells in vivo, which will be significant in both cancer therapy and aging prevention.

CHAPTER 2

METHODS

2.1 Cell Culture and Cell Lines

Li-Fraumeni Syndrome (LFS) is an inherited cancer syndrome caused by germline mutations in the tumor suppressor p53. Patients with this syndrome are prone to several types of cancers, including breast cancers, soft tissue sarcomas, brain tumors, osteosarcomas, leukemias, lymphomas and adrenocortical carcinoma [73]. Dermal fibroblasts from these patients were established in cell culture, and characterized [74]. It was found that though these fibroblasts can grow normally and senesce due to telomere erosion as a normal cell would, they could also lose their functional copy of p53 due to genomic instability and spontaneously immortalize [7]. The immortal cells obtained included MDAH041, MDAH172, MDAH174 as well as four independent immortalizations of the same cell line: MDAH087-N, MDAH087, MDAH087-1 and MDAH087-10. These immortal cells show altered morphology, as well as chromosomal anomalies and can be transformed by oncogenes to form tumors [7]. It is important to note that fibroblasts from normal patients or other cancer predisposing syndromes do not spontaneously immortalize in culture. MDAH041 early passage cells possess one allele with a frameshift mutation at amino acid 184 of p53, which results in a truncated p53 protein with no function. Spontaneously immortalized MDAH041 cells subsequently lose their wild-type copy of p53 and therefore have no copies of functional p53 [74]. MDAH041 cells contain active telomerase and therefore maintain short but stable telomere lengths. MDAH172 and MDAH174 cells have a missense mutation at codon 175 of p53, and the MDAH087 cells have a missense mutation at codon 248 of p53 [74]. MDAH172,

MDAH174 and MDAH087 immortal cells do not have active telomerase but maintain long telomeres through the alternative lengthening of telomeres (ALT) mechanism [74, 75].

MDAH041 cells were derived from primary fibroblasts by skin biopsy from an LFS patient and were spontaneously immortalized *in vitro*. Immortalized LFS MDAH041 fibroblasts, as well as the 3 other LFS cell lines: MDAH087-1, MDAH087-N and MDAH172 were grown in Modified Eagles Medium (MEM, Invitrogen). Cells were supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) and were maintained at 37°C in 5% humidified CO_2. Cells were maintained through passage from 1 plate to 4 plates every 3 days.

2.2 Senescence-Inducing Drug Treatments

H_2O_2 treatment was performed by plating immortal LFS cells at $4x10^5$ cells per 10 cm plate and adding H_2O_2 to cell media at a final concentration of 20 μM for MDAH-087-N and MDAH087-1, 25 μM for MDAH172 and 85 μM for MDAH041 cells and incubating at 37°C for two hours in 5% CO_2. Optimal concentration of H_2O_2 for each cell line was determined by greatest number of SA- β-galactosidase positive cells. Plates were then washed with PBS and replenished with growth media, and incubated at 37°C for 5 days.

5-aza-2-Deoxycytidine treatment was performed by plating immortal LFS cells at $3x10^5$ cells per 10 cm plate, adding fresh preparations of sterile 5-aza in 50% acetic acid to cell media to a final concentration of 1 μM every other day and plates were harvested on day 8.

Adriamycin treatment was performed by plating immortal LFS cells at $4x10^5$ and adding a stock of adriamycin dissolved in water to cell media at a final concentration of

0.50 μM for MDAH041 cells and 0.20 μM for MDAH087-1, 087-N and 172 cells. Optimal concentration of Adriamycin for each cell line was determined by greatest number of SA-β-galactosidase positive cells. Cells were treated for two hours (MDAH041) or 1.5 hours (087-N, 087-1 and 172) at 37 °C, were washed with PBS and growth media was replenished. Cells were grown at 37°C for 5 days before harvest.

Proliferating cells were harvested at a lowest population doubling (PD 10-12), low population doubling (PD 18-20) and one plate of proliferating cells was kept to expand for naturally senescent cells of the same line. Naturally senescent cells were achieved through serial passaging at a 1 to 2 split until cells halted proliferation and appeared morphologically senescent (PD 29-30). Immortal cells were employed at high population doublings (greater than 200). Quiescent cells were obtained by plating immortal cells at $3x10^5$ cells per 10 cm plate, washing the next day with PBS, and adding media with 0.1% serum. Cells were then incubated for 24 hours and harvested.

2.3 Real-Time PCR and Primers

cDNA was prepared from 3 μg of RNA, using the Superscript II system from Invitrogen. Q-RT-PCR was performed using Power SYBR Green MasterMix from Applied Biosystems and analyzed on the ABI 5700 Sequence Detection System (Applied Biosystems). Primers for each gene analyzed are listed in Table One. The relative fold change was calculated using the C_T method as follows: $2^{-\Delta\Delta CT}$, where, $\Delta\Delta C_T = (C_{T\ Gene\ of\ interest} - C_{T\ GAPDH})_{experiment} - (C_{T\ Gene\ of\ interest} - C_{T\ GAPDH})_{control}$. Statistical significance was determined through student's t-test and a p-value of less than 0.05 was considered significant.

IL1A Forward	CTTAAGCTGCCAGCCAGAGA
IL1A Reverse	ACCAAACCAGGGAGGGACAA
IL1B Forward	CCAGCTACGAATCTCCGACC
IL1B Reverse	CATGGCCACAACAACTGACG
Myd88 Forward	CCTCAAGTCCTGGGGAAATGC
Myd88 Reverse	AAGGCTCAGGAGACCCACTG
IL8 Forward	GAGACAGCAGAGCACACAAG
IL8 Reverse	GATGTGCTTACCTTCACACAGA
ICAM1 Forward	GGTAGCAGCCGCAGTCATAA
ICAM1 Reverse	TCCCTTTTTGGGCCTGTTGT
IRF5 Forward	TTCTCTCCTGGGCTGTCTCTG
IRF5 Reverse	CTATACAGCTAGGCCCCAGGG
IRF7 Forward	GCAGCGTGAGGGTGTGTCTT
IRF7 Reverse	GCTCCATAAGGAAGCACTCGAT
Cyclin A2 Forward	AGTGATGTTGGGCAACTCTG
Cyclin A2 Reverse	TCCGGGTTGATATTCTCCTG
GAPDH Forward	ATCAAGAAGGTGGTGAAGCAG
GAPDH Reverse	TGTCGCTGTTGAAGTCAGAGG

Table 1: Primers used for RT-PCR

2.4 RNA Extraction

RNA was extracted from 10 cm plates of 80% confluent cells of all conditions using the QIAGEN RNeasy Kit (QIAGEN). Fully supplemented media was added to the cells approximately 18 hours before harvest to ensure cell cycle participation.

2.5 Immunocytochemistry and Antibodies

Cells were plated in 2-well chamber slides (Thermo-Scientific). Cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, washed three times with PBS and permeabilized with 0.1% Triton for 15 minutes. Slides were then washed three times with PBS and blocked with 0.2% BSA for 30 minutes and incubated with primary antibody suspended in 0.2% BSA for 1 hour at room temperature. Following three PBS washes, slides were incubated with appropriate secondary antibodies suspended in 0.2% BSA for one hour at room temperature. Three washes with PBS were performed and excess PBS was allowed to evaporate from the chamber before Prolong Gold antifade reagent with DAPI (Life Technologies) was added to the slide, and covered with a coverslip. Antibodies used are listed below.

Antibody	Company	Catalog Number
IL1β	R&D	MAB601
IL1α	R&D	MAB200
Anti-Mouse FITC	Santa Cruz	SC-2099
Anti-Mouse TRITC	Santa Cruz	SC-2981

Table 2: Antibodies used for Immunocytochemistry

2.6 Senescence-Associated β-Galactosidase Staining

A senescence detection kit (BioVision) was used for staining according to the manufacturer's instructions. Briefly, treated cells were stained for senescence-associated β-galactosidase activity after 5-7 days of drug treatment. In order to count the senescence-associated β-galactosidase positive cells, cell plates were washed twice with PBS and fixed with fixative solution for 10-15 minutes at room temperature. The fixed cells were washed with PBS and stained with the staining solution containing X-gal and staining supplement and incubated at 37°C overnight with no CO_2. Cells containing blue stain were counted as senescent, proportionate to the total number of cells per field of vision. At least 3 fields of vision were counted, with a minimum of 200 cells per plate. Statistical significance was determined through student's t-test and a p-value of less than 0.05 was considered significant.

2.7 Cell Cycle Analysis

Cell cycle analysis was performed using propidium iodide staining. MDAH041 immortal cells were plated at 50% confluence. On the next day, they were rinsed 3 times with PBS and given media supplemented with 0.1% FBS. A control plate of immortal cells was also included which were supplemented with 10% FBS. After 48 hours, cells were trypsinized and rinsed twice with PBS. Pellet was resuspended in 1 mL PBS and added dropwise to 1 mL ice cold 95% ethanol, and stored overnight at 4°C. Cells were rinsed twice with PBS, then pelleted again and resuspended in 500 uL staining solution: 10 mL 0.1% Triton x-100 in PBS with 0.4 mL 500 ug/mL propidium iodide and 25 uL 10 mg/mL RNAase A and incubated for 37 ^{o}C for 15 minutes. After incubation, tubes were transferred to 4 ^{o}C and protected from light until flow cytometry was performed by the flow cytometry core at Wayne State University.

2.9 RNA-sequencing and Data Analysis

Quality control for all RNA samples was performed on the Agilent 2100 Bioanalyzer by the Applied Genomics Technology Center at Wayne State University. Measurements include RIN (RNA integrity number) and 28S/18S ratio. RIN measured the degradation of RNA, as well any potential contamination with genomic DNA, and values close to 10 are optimal. The 28S/18S ratio is measured through electrophoresis, and the ratio should be close to 2. The size ratio of the 28S/18S ribosome is technically 2.7:1, or 5kb:2kb but 2:1 is the benchmark for intact RNA.

Preparation of samples for sequencing was done at the AGTC using the TruSeq RNA sample preparation kit (Illumina). Briefly, mRNA is taken from the total RNA by magnetic beads containing T oligos, which bind the 3' poly A tails of mRNA, separating it from the total RNA. The samples are fragmented by divalent cations and elevated temperature and reverse transcribed into cDNA. The ends are repaired by addition of an "A" base and ligated to adapters which enable multiplexing of samples, and all fragments are enriched with PCR to get a final cDNA library. In this sequencing run, the samples were multiplexed with 6 samples per lane, in 4 lanes, for a total of 24 samples.

Samples were then sequenced on the Illumina HiSeq 2000 System, and analyzed by Dr. Adele Kruger at the AGTC. Briefly, samples were demultiplexed with Illumina's CASAVA 1.8.2 software (www.illumina.com) and quality control was assessed using FastQC from Babraham Bioinformatics (www.bioinformatics.babraham.ac.uk/). Reads were then aligned to the Human Genome Consortium's reference human genome hg19 [76] using Tophat software [77], allowing 20 alignments to the genome per sequencing read. Relative abundances and differential expression was calculated with Cufflinks [78].

Briefly, the Cuffdiff 2 feature of Cufflinks uses an algorithm that identifies differentially expressed genes by testing an observed fold change (calculated from FPKM values of different samples) against a null hypothesis (no change). To account for variability, this program uses a model of variability which analyzes significance, through a table that predicts variance in number of gene reads for both conditions that are being compared. This algorithm eventually yields an estimate of the number of reads for each gene and a variance for that estimate, which are reported along with FPKM values and corresponding variance. Differential expression if shown as fold change of FPKM, and the variance of FPKM allows the program to calculate variance for the fold change itself. Therefore, a gene with variable expression will have a more variable fold change, and these variables are reflected in the resulting p-value.

Pathway analysis was performed using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com), Signaling Pathway Impact Analysis (SPIA) [79] with the help of Calin Voichita in the Computer Science Department, and Genomatix Genome Analyzer.

Clustering analysis was performed using Cluster 3.0 software and Treeview software. A hierarchical clustering was performed after log transforming the gene expression data (FPKM values or relative abundance). The center median of the gene values were taken, and both the genes and arrays were clustered with an uncentered correlation and average linkage. Results are presented as relative up-regulation or down-regulation compared to the median expression of a given gene.

CHAPTER 3

RESULTS

3.1 Analysis of Samples for RNA-seq and RNA-seq

Eight RNA samples were obtained for potential RNA-seq: immortal cells (PD>200), lowest passage cells (PD 10-12), low passage cells (PD 17-19), replicatively senescent cells (PD 28-30), quiescent cells (PD>200, serum-starved), H_2O_2-induced senescent cells (PD>200, treated with H_2O_2), adriamycin-induced senescent cells (PD>200, treated with adriamycin), and 5-aza-induced senescent cells (PD>200, treated with 5-aza). In order to perform RNA-seq, I first needed to confirm that the cells were senescent and the samples were high enough quality for sequencing.

Senescence-associated β-galactosidase staining was performed on simultaneous plates grown in parallel with those harvested for RNA to assess the amount of senescence in all samples (Figure 1). Cell cycle analysis was performed on the quiescent samples to confirm withdrawal from the cell cycle (Figure 2). These RNA samples were transported to the Applied Genomics Technology Center, where quality control was performed to assess the amount of degradation of RNA and any potential contamination with genomic DNA (Figure 3). After quality was ensured, the RNA was prepared for sequencing.

In addition to quality control analysis performed by the Applied Genomics Technology Center, RT-PCR was performed on genes known to change during cellular senescence based on previous studies. Cyclin A was shown to decrease in senescent samples, consistent with withdrawal from the cell cycle (Figure 4). Additionally, interferon regulatory factors IRF3, IRF5 and IRF7 were examined to analyze the participation of the

interferon pathway in all types of senescence (Figures 5, 6, 7). IRFs are transcription factors that can activate several members of the interferon pathway [80].

3.1.1 Senescence-Associated β-Galactosidase Staining of RNA-seq Samples

Senescence-associated β-galactosidase staining was performed on all samples concurrent to RNA extraction (Figure 1). All 4 types of senescence (natural, Adriamycin-induced, 5-aza-induced and H_2O_2-induced) exhibited high levels of senescence-associated β-galactosidase positive cells compared to the immortal and quiescent samples, which had only very low baseline levels of senescence-associated β-galactosidase staining. The difference in senescence-associated β-galactosidase staining in the senescent samples was statistically significant compared to the immortal samples with $p<.05$ using a student's t-test. Proliferating lowest passage cells and low passage cells showed a low level of senescence. Three biological replicates were used for each condition. This indicates that the 4 types of senescence were indeed senescent while the other conditions (immortal, quiescent, low passage, and lowest passage), were not senescent.

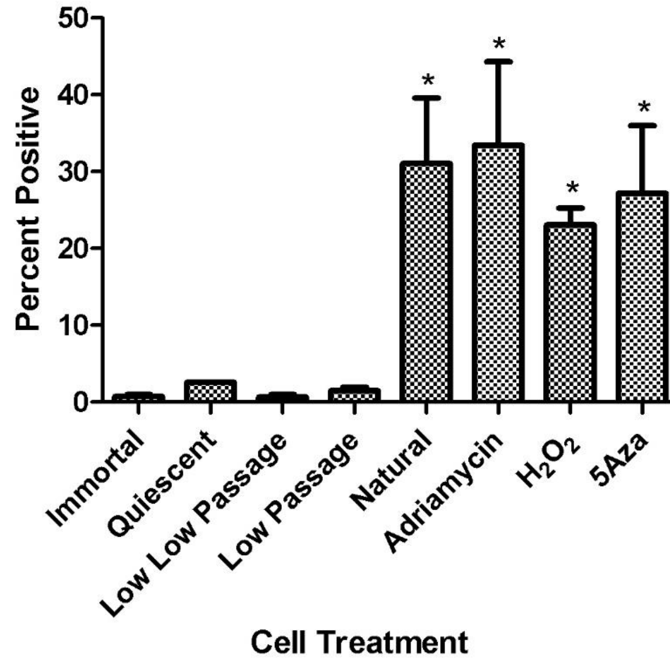


Figure 1: Senescence-Associated β-galactosidase Activity Levels in RNA-seq Samples. All 4 types of senescence show a significantly higher level of SA-β-gal staining compared to the immortal, quiescent, lowest passage, and low passage samples. Bars indicate percentage of total cells that are senescence-associated β-galactosidase staining positive. Error bars represent three biological replicates. Statistical significance marked by astericks.

3.1.2 Cell Cycle Analysis of Quiescent Samples

Cell cycle analysis was performed through propidium iodide staining to confirm cell cycle arrest in quiescent samples (Figure 2). A plate of cells growing simultaneously to the plates harvested for RNA-seq was used for propidium iodide staining. The quiescent samples showed increased levels of G1 and decreased levels of S phase compared to the proliferating immortal control, consistent with cell cycle arrest at G1/S. Therefore these cells were confirmed to be quiescent and suitable for RNA-seq.

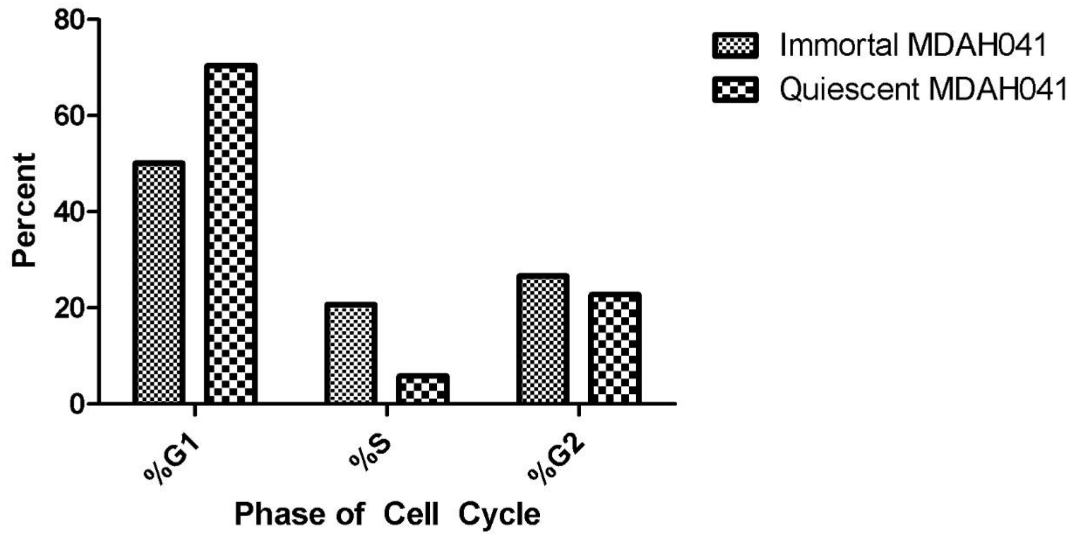


Figure 2: Cell Cycle Analysis of Quiescent Samples. The quiescent MDAH041 cells show increased %G1 and decreased %S phase, indicating an accumulation of cells at G1/S, representing quiescence. Dotted bars indicate immortal samples, squared bars indicate quiescent samples. Results are shown as percentage of cells in each G1, S or G2 phase via propidium iodide flow cytometry analysis.

3.1.3 Quality Control of RNA-seq Samples

Quality control was performed on all 24 samples used for RNA-seq (8 experimental cell conditions, in biological triplicate, to yield 24 samples). Optimal samples have RIN (RNA integrity number) values close to 10 and 28S/18S ratios close to 2, as described in section 2.9). All samples under consideration for RNA-seq exceeded values for quality control, with the exception of sample 8C which had a lower RIN than the rest of the samples, but was still deemed acceptable (Figure 3). Therefore, all samples were then used for RNA-seq. Sample 8C was later shown to have consistent gene expression levels with samples 8A and 8B, which validated the decision to include the sample in analysis.

		RIN	28S/18S
1a	H_2O_2	9.8	2.1
1b	H_2O_2	10	1.9
1c	H_2O_2	9.8	1.7
2a	Imm	10	2.3
2b	Imm	10	2
2c	Imm	10	1.8
3a	LP	10	1.9
3b	LP	9.9	1.8
3c	LP	9.7	1.9
4a	Adria	9.5	1.8
4b	Adria	8.5	1.6
4c	Adria	9.6	1.7

		RIN	28S/18S
5a	nat	9.8	1.9
5b	nat	9.6	1.6
5c	nat	9.6	1.7
6a	5Aza	9.5	1.5
6b	5Aza	9.7	1.5
6c	5Aza	9.6	1.6
7a	Quies	10	2.1
7b	Quies	10	1.9
7c	Quies	9.9	2
8a	LLP	10	1.8
8b	LLP	9.9	2
8c	LLP	6.6	1.2

Figure 3: RNA Integrity of RNA-seq Samples. RIN (RNA Integrity Number) and 28S/18S ratios are shown for each biological replicate of each condition. Sample 8c was the only sample with a low RIN, but the RIN was not low enough to exclude the sample from sequencing.

3.1.4 Cyclin A Expression in RNA-seq Samples

To further confirm that the 4 types of senescent cells were senescent, cell cycle withdrawal through RT-PCR analysis of Cyclin A was performed on all senescence types (Figure 4). Previous studies in the Tainsky laboratory have shown that cyclin A expression decreases in various types of senescence because of cell cycle withdrawal that is incidental during senescence [36]. Cyclin A was down-regulated, in all 4 types of senescent cells compared to the proliferating immortal control. This was consistent with previous studies, confirming these cells were senescent.

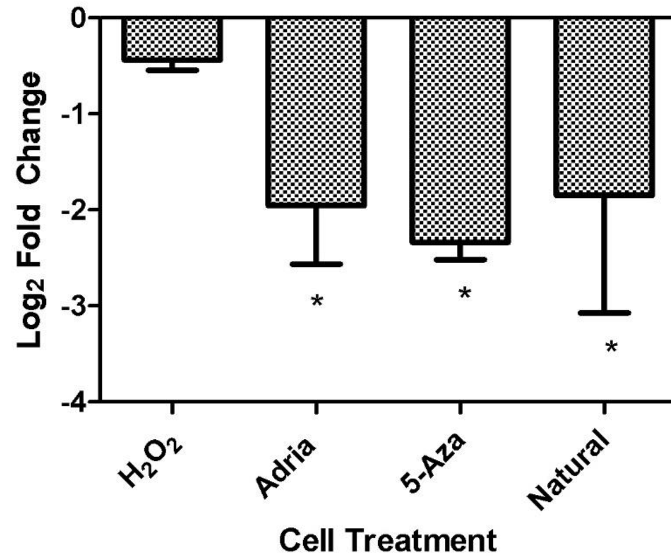


Figure 4: Cyclin A Expression in RNA-seq Samples. All 4 types of senescent cells showed a decrease in Cyclin A2 expression compared to the immortal control. Bars indicate RT-PCR $\log_2$ fold change of cyclin A compared to immortal cells. Statistically significant changes relative to the immortal sample are marked with astericks. Error bars indicate three biological replicates.

3.1.5 Interferon Regulatory Factor Expression in RNA-seq Samples

Based on previous data from the Tainsky lab indicating the Interferon pathway is up-regulated during cellular senescence, RT-PCR analysis was performed on IRF3, IRF5 and IRF7 from all senescence types to ascertain whether the interferon pathway was up-regulated in all types of senescence (Figures 5, 6, 7). IRF3 was down-regulated in H_2O_2 and Natural senescence, and up-regulated in 5-aza-induced senescence (Figure 5). In the RNA-seq data, IRF3 was not shown to be differentially expressed in any type of senescence compared to immortalization. IRF5 was universally up-regulated compared to the immortal control, confirming a correlation of the interferon pathway and senescence (Figure 6). In the RNA-seq data, IRF5 was not shown to be differentially expressed in any type of senescence compared to immortalization. IRF7 showed up-regulation in 5-aza-induced cells and natural senescence (Figure 7). In the RNA-seq data, IRF7 was up-regulated in natural senescence and 5-aza-induced senescence compared to the immortal control. Additionally in the RNA-seq data, IRF9 was up-regulated in all 4 types of senescence compared to immortalization, however it was also shown to be up-regulated in quiescence at a similar level compared to immortalization, indicating this IRF may have a cell cycle related function. RNA-seq data also showed IRF1 to be up-regulated in Adriamycin-induced, H_2O_2-induced and 5-aza-induced senescence compared to immortalization. Each type of senescence exhibited a different profile of IRF expression, indicating a universal correlation of the interferon pathway in senescence but potential diversity of these interferon pathways in the various types of senescence.

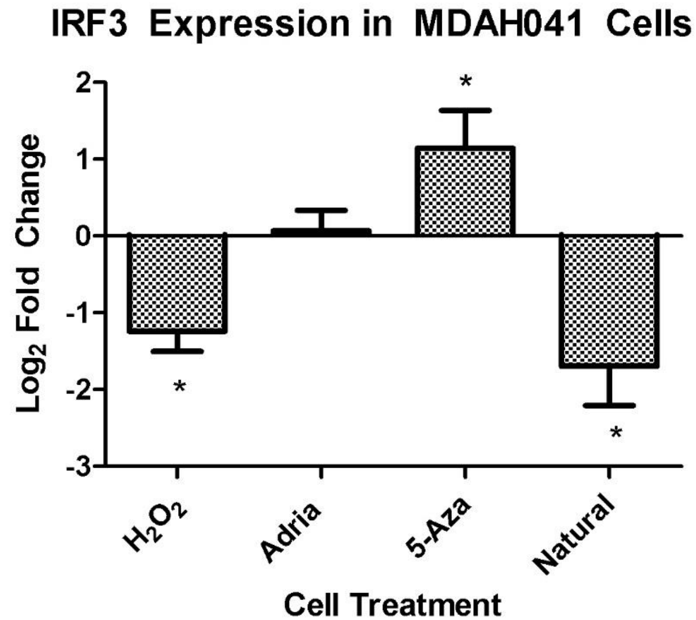


Figure 5: Interferon Regulatory Factor 3 Expression in RNA-seq Samples. IRF3 is down-regulated in H_2O_2-induced and naturally senescent cells, compared to immortal cells. Adriamycin shows no difference in IRF3 compared to immortal cells, and 5-aza-induced senescence shows up-regulation of IRF3 compared to immortal cells. Bars indicate RT-PCR log_2 fold changes of IRF3 expression relative to the immortal sample. Statistically significant changes from the immortal control are marked by astericks. Error bars indicative of standard deviation of 3 biological replicates.

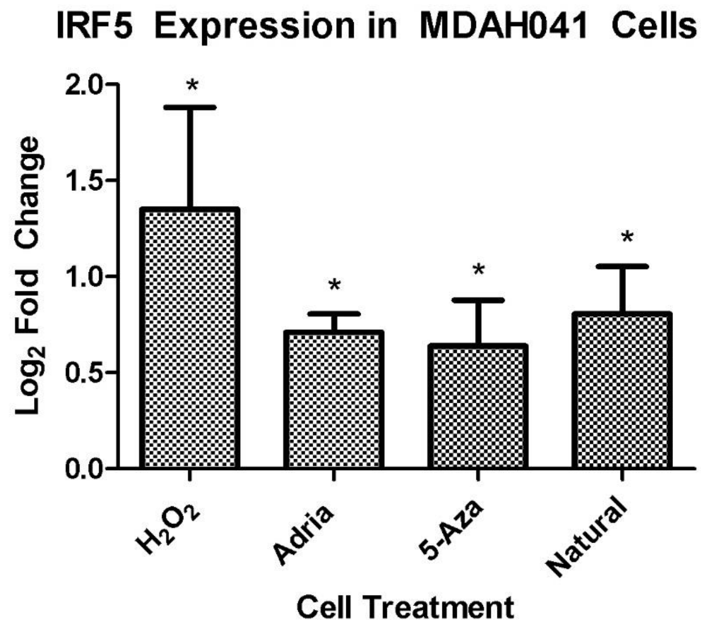


Figure 6: Interferon Regulatory Factor 5 Expression in RNA-seq Samples. IRF5 is up-regulated in all 4 types of senescent cells compared to immortal cells. Bars indicate RT-PCR log_2 fold changes of IRF5 expression relative to the immortal sample. Statistically significant changes from the immortal control are marked by astericks. Error bars indicative of standard deviation of 3 biological replicates.

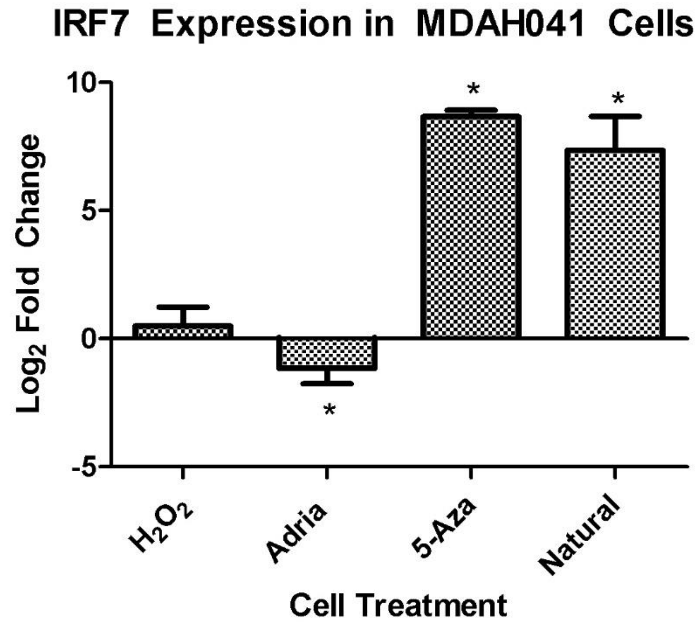


Figure 7: Interferon Regulatory Factor 7 Expression in RNA-seq Samples. IRF7 is up-regulated in 5-aza-induced and naturally senescent cells compared to immortal cells. H_2O_2 doesn't show a significant different in IRF7 expression compared to immortal cells. Adriamycin-induced senescence shows down-regulation of IRF7 compared to immortal cells. Bars indicate RT-PCR $\log_2$ fold changes of IRF7 expression relative to the immortal sample. Statistically significant changes from the immortal control are marked by astericks. Error bars indicative of standard deviation of 3 biological replicates.

3.2 RNA-seq Analysis and Clustering

RNA was sequenced as described in the methods section. First, differentially expressed genes in all types of senescence compared to the immortal control were examined. Differentially expressed genes were those that had a +/-2 fold change relative to the control with a p-value of less than 0.05. The intersection of all 4 types of senescence, or genes that were differentially expressed in all 4 types of senescence yielded 93 genes (Figure 8). However, the genes that were differentially expressed in quiescent cells compared to the immortal control were removed from this population of genes in an effort to remove the number of genes that were differentially expressed only due to withdrawal from cell cycle (quiescence) during senescence leaving those that were senescence-specific. Therefore, 45 genes were subtracted from the population, leaving a final list of 48 genes that were differentially expressed in all 4 types of senescence compared to immortalization, but not differentially expressed in quiescence compared to immortalization.

Pathway analysis was performed using a variety of programs, including Genomatix Genome Analyzer (GGA), Ingenuity Pathway Analysis (IPA) and Signaling Pathway Impact Analysis (SPIA). SPIA analysis was performed by collaborators in the Computer Science Department. Initially, Genomatix analysis showed the most probable pathways involved with senescence to be the immune system and interferon related pathways, which is consistent with previous results from the Tainsky laboratory. To confirm these results, IPA analysis was also performed. This type of analysis gave the same results, indicating the immune system and interferon pathways to play a large role in senescence based on the 48 genes studied and showing it is not a software-dependent finding.

Clustering analysis was performed using Cluster 3.0 software and Treeview software (Figure 9, 10, 12, 13, 16) to compare patterns of gene expression among various samples. This type of analysis showed that the 4 types of senescent cells had different gene expression profiles than immortalization, and that the quiescent cells showed a different gene expression profile than the senescent cells. Additionally, a progression from lowest passage cells to low passage cells to natural senescence can be observed in the form of progressive up-regulation of certain genes associated with senescence.

3.2.1 Comparison of Differentially Expressed Genes

5-aza-induced senescent cells had 3990 genes that were differentially expressed compared to the immortal control (Figure 8). 2449 were up-regulated and 1350 were down-regulated. The adriamycin-induced group had 1425 genes that were differentially expressed compared to the immortal control, where 1067 genes were down-regulated and 358 were up-regulated. Naturally senescent had 2729 genes that were differentially expressed compared to the immortal cells, 1379 down-regulated and 1350 up-regulated. H_2O_2 senescent cells had 193 genes that were differentially expressed, 14 down-regulated and 179 up-regulated. The intersection of these 4 conditions yielded a list of 93 genes that were differentially expressed in all 4 types of senescence. Forty-five genes that were differentially expressed in quiescent cells compared to immortal cells were subtracted from the 93 genes in order to account for changes due only to the withdrawal from cell cycle. This yielded a total of 48 genes that were differentially expressed in all four types of senescence but not in quiescence.

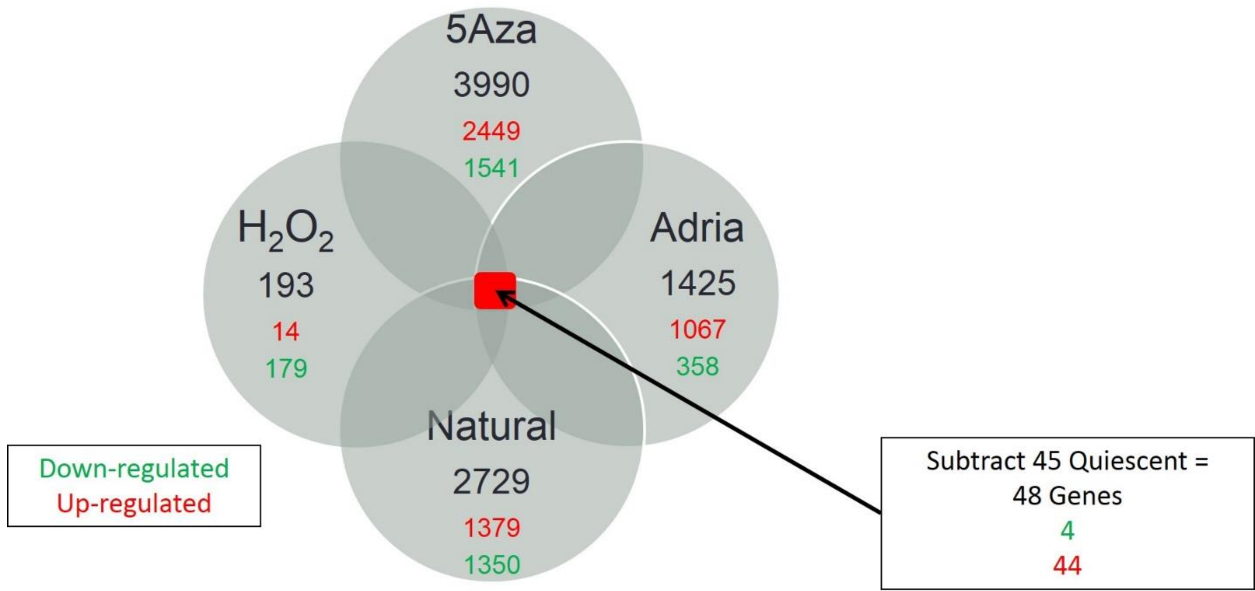


Figure 8: Comparison of Differentially Expressed Genes. The differential gene expression in 4 types of senescence compared to immortalization. Black numbers indicate number of differentially expressed genes between the given sample and immortal sample, red number indicates number of genes up-regulated in the given sample compared to the immortal sample, and green numbers indicate number of genes down-regulated in the given sample compared to the immortal control. Dark gray portions indicate overlap between samples, and the red center indicates overlap between all 4 types of senescence. Red numbers show up-regulated genes and green show down-regulated genes.

3.2.2 Clustering Analysis of 45 Genes Differentially Expressed in Quiescent and Senescent RNA-seq Samples

Clustering analysis of the 45 genes differentially expressed in quiescent cells, immortal cells as well as all 4 types of senescent cells was performed. Interestingly, the quiescent gene expression was most similar to the immortal gene expression and very different from the senescent gene expression, which reinforced the validity of removing the quiescent genes from senescence-associated pathway analysis (Figure 9).

The 45 genes were also clustered with the immortal sample being compared to the low passage and lowest passage samples, as well as natural senescence (Figure 10). The immortal sample associated closest to the lowest passage sample, which is consistent with the idea that these genes are down-regulated during cell growth and up-regulated during senescence. The low passage sample which are proliferating at a slower rate due to impending senescence associated closest to natural senescence, again indicating that these genes are up-regulated as a cell ages.

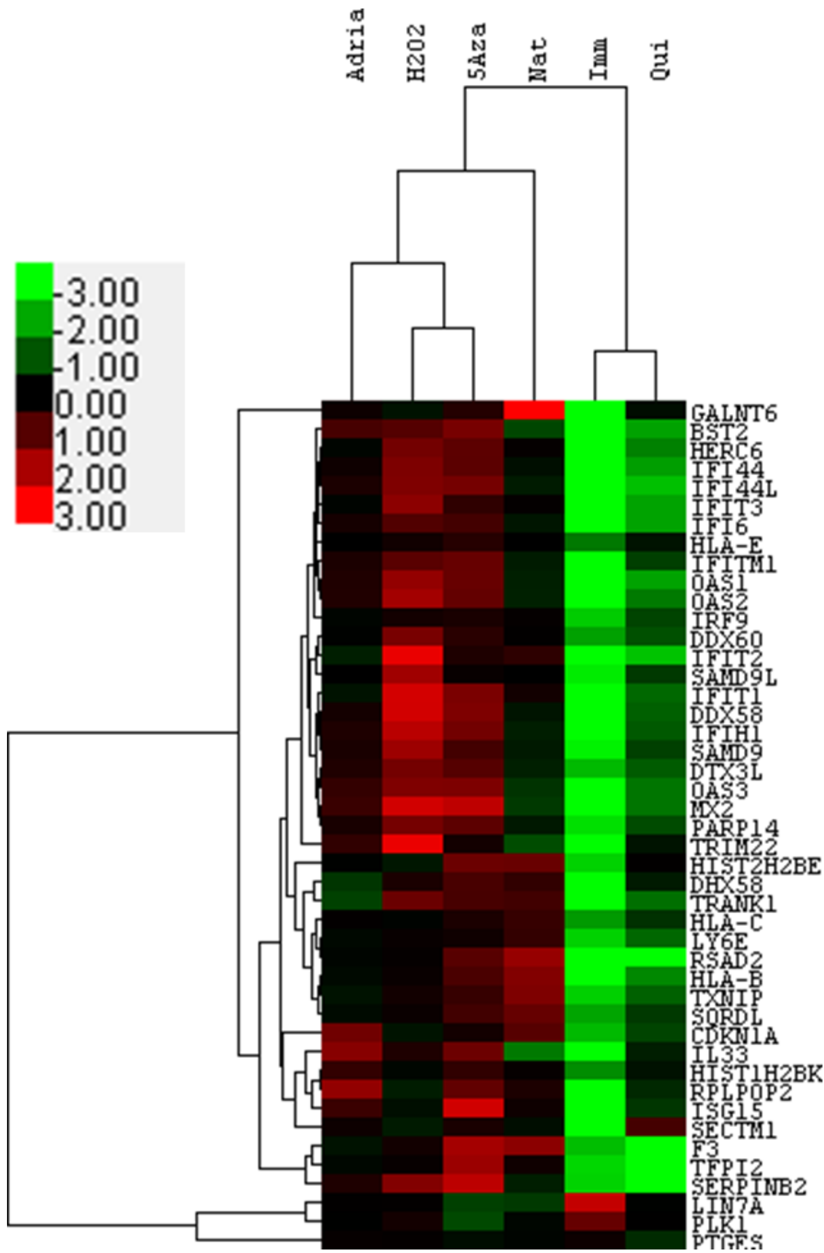


Figure 9: Clustering Analysis in Immortalization and Senescence of the 45 Quiescent-Associated Genes. The 45 quiescence-associated genes are mostly up-regulated in all 4 types of senescence and down-regulated in quiescence and immortalization. Green bars show relative down-regulation and red shows relative up-regulation compared to the median expression of a given gene. Expression from all 4 types of senescence, quiescence and immortalization are shown.

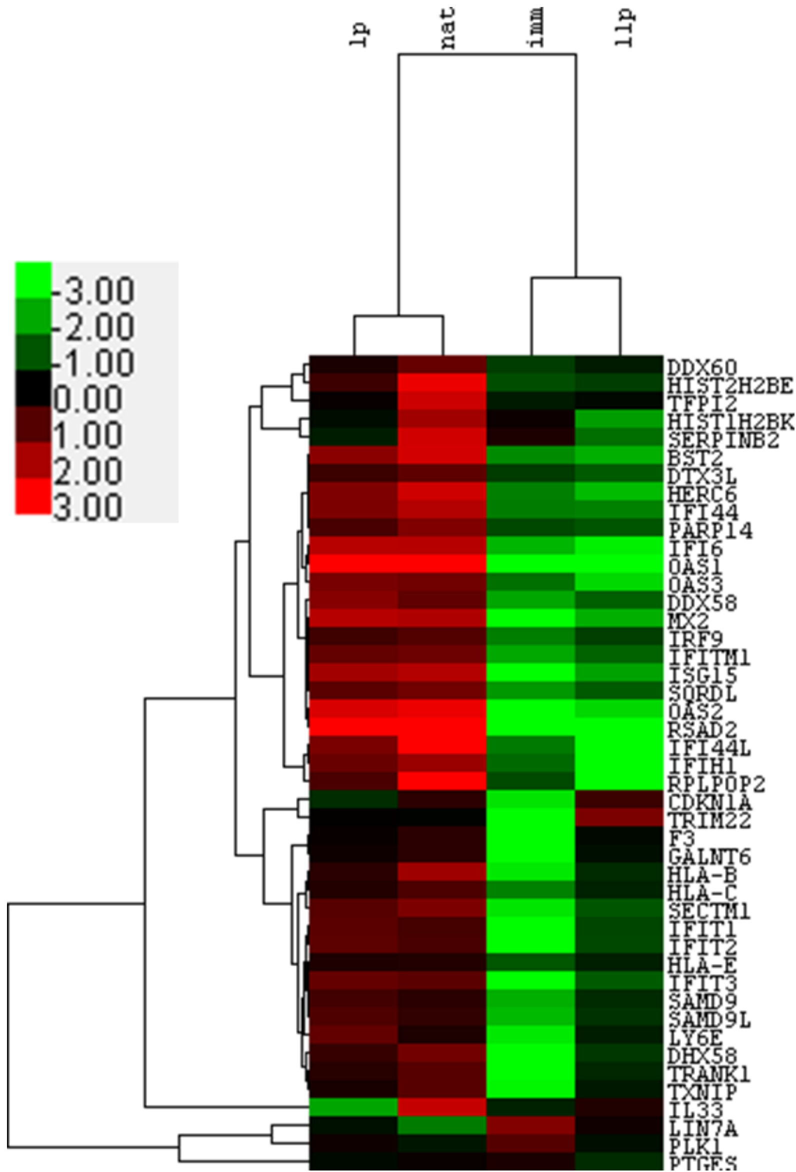


Figure 10: Clustering Analysis in Immortalization and Aging Samples of the 45 Quiescent-Associated Genes. The 45 quiescence-associated genes were mostly up-regulated in low passage aged cells and naturally senescent cells, and mostly down-regulated in quiescent cells and immortal cells that are still proliferating. The genes were clustered to show comparison of the genes between immortal, low passage, lowest passage and natural senescence. Green bars show relative down-regulation and red shows relative up-regulation compared to the median expression of a given gene.

3.2.3 Forty-Eight Genes Differentially Expressed in all 4 Types of Senescence

There were 48 genes that were deemed senescence-associated based on their differential expression in all 4 types of senescence compared to immortalization and absence of differential expression in quiescence compared to immortalization (Figure 11). Clustering was first performed on the 48 genes found to be differentially expressed in all four types of senescence compared to immortalization (Figure 12). This showed an inverse relationship between senescence and immortalization, which was expected. The genes that were highly up-regulated in all 4 types of senescence were down-regulated in immortalization, and the genes that were highly up-regulated in immortalization were down-regulated in all 4 types of senescence. 5-aza-induced senescence shows a higher up-regulation of most genes compared to the other types of senescence. This data shows the inverse relationship between senescence and immortalization and further confirms the validity of this RNA-seq dataset.

Additionally, these 48 genes were clustered for early lowest passage cells and low passage cells, as well as natural senescence and immortalization in order to analyze the relationship (Figure 13).This showed the closest relationship between immortalization and early low passage cells, which is not surprising seeing that both sets of cells are proliferating. Additionally, the natural senescent cells were most closely associated with the low passage cells, which is not surprising seeing that the low passage cells are aged and approaching senescence, therefore not proliferating very much. Additionally, a progressional up-regulation of genes from lowest passage, to low passage, to natural senescence can be observed which indicates that several senescence-associated genes become increasingly activated as the cell ages.

APOL1	EPSTI1	LCP1
APOL3	GBP4	LGALS9
ATF3	GCA	MIR4632,TNFRSF1B
BATF2	GMPR	MLKL
BIRC3	GNG11	MMP12*
C3	HERC5	MYD88
C9orf47,S1PR3	ICAM1*	NEGR1
CCDC80	IDO1	OASL
CD163L1	IFI27	ODZ2
CD82	IFI30	PLAU
CLDN1	IL1A*	PMAIP1
COL17A1,MIR936	IL1B*	RSPO3
CTSS	IL1RN	RTP4
CXCL2*	IL32	SCN3A
CYP1A1	ISG20	TNFAIP3
DUSP6	ITGB3	TNFAIP6

Figure 11: 48 Genes Senescence-Associated Genes. 48 genes were deemed senescence-associated because they were differentially expressed in all 4 types of senescence compared to immortalization, but not differentially expressed in quiescence compared to immortalization. After pathway analysis indicated a role for immune system pathways in senescence, the list was updated to show genes related to the immune system in red. Additionally, genes listed with an asterick indicate genes that are part of the senescence-associated secretory phenotype.

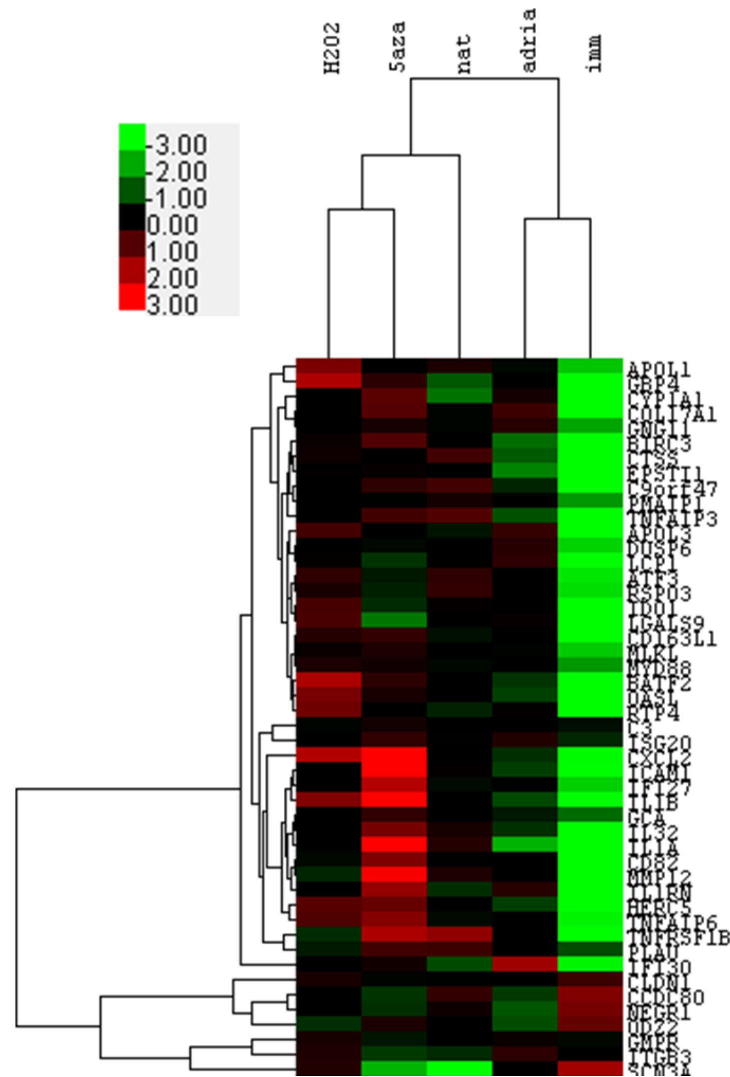


Figure 12: Clustering Analysis in Immortalization and Senescence of the 48 Senescence-Associated Genes. Immortal cells show an inverse relationship compared to the 4 types of senescence. Genes down-regulated in immortalization are up-regulated in senescence, and genes up-regulated in immortalization are down-regulated in senescence. Immortal sample and all 4 types of senescence are shown here. Green bar indicates relative down-regulation and red bars indicate relative up-regulation relative to the median expression of a given gene.

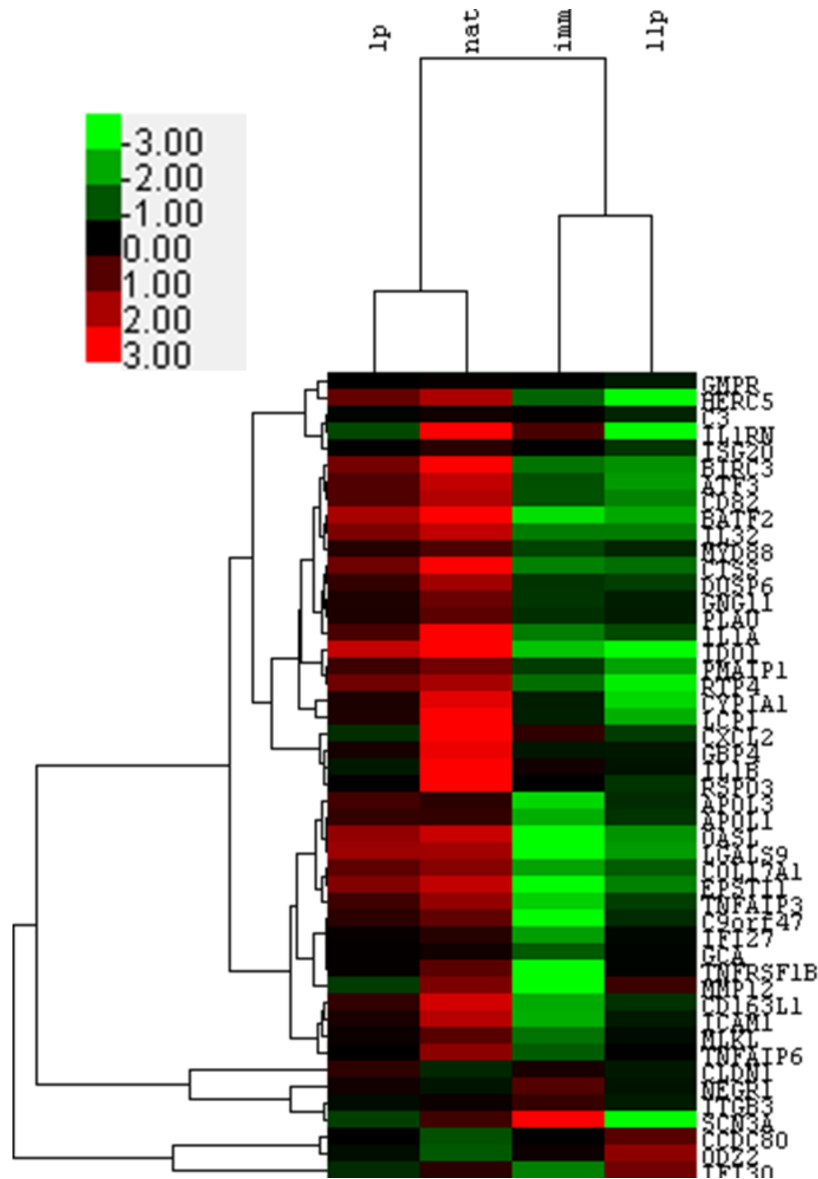


Figure 13: Clustering Analysis in Immortalization and Aged Samples of 48 Senescence-Associated Genes. Most of the 48 senescence-associated genes become increasingly up-regulated as a cell ages and approaches senescence. Immortal sample, low passage, lowest passage and natural senescent samples are shown here. Green bars indicate relative down-regulation and red bars indicate relative up-regulation compared to median expression of a given gene.

3.3 The Interferon/Inflammatory Pathway is Differentially Expressed in 4 Types of Senescence

After identification of the 93 genes that were differentially expressed in all types of senescence, the 45 genes that were also differentially expressed in quiescence were subtracted to yield a total of 48 genes that were differentially expressed in all 4 types of senescence but not associated with quiescence. Cluster analysis from section 3.2 (Figure 9) validated the decision to remove the 45 genes from senescence-associated pathway analysis because the quiescent sample associated most closely to the immortal sample for these 45 genes, indicating that the quiescent samples were different from the senescent samples. The resulting 48 genes (Figure 11) after subtraction of the 45 quiescent genes were used for pathway analysis through Genomatix Genome Analyzer and Ingenuity Pathway Analysis.

Pathway analysis through both types of software indicated immune system pathways to be highly significant for these 48 genes, indicating a possible role for the immune system/inflammatory pathways in all 4 types of senescence (Tables 3 and 4). This finding is consistent with previous data from the Tainsky laboratory, also showing a role for the Interferon/Inflammatory pathway in senescence. All genes from the interferon pathway and IL1 pathway, shown below, were up-regulated in the dataset in all 4 types of senescence compared to immortalization. A schematic of the genes included in the "inflammation" pathway according to Genomatix is shown in Figure 14. All of the genes within this pathway were up-regulated in senescent cells. Figure 15 shows a schematic of the "defense response" pathway according to Genomatix. All genes in this pathway were also up-regulated in senescent cells compared to immortal cells.

Because of previous Tainsky lab data showing a role for the interferon regulatory factors in 5-aza-induced senescence, I analyzed the presence of IRFs in all 4 types of senescence through clustering analysis (Figure 16). The 5-aza-induced senescence sample associated similarly with the natural senescenct sample, indicating a similarity between the two types of senescence. The analysis also showed a down-regulation of most IRFs in immortalized cells compared to the 4 types of senescent cells, which was expected due to the increased activity of IRFs in 5-aza-induced senescence that the Tainsky lab previously showed. The 5-aza-induced senescence sample exhibited an up-regulation of IRF6 and IRF7, which is consistent with RT-PCR shown above which shows high up-regulation of IRF7 in this sample. Additionally, natural senescence showed high expression of IRF4 and IRF7, which again is consistent with above RT-PCR data showing high levels of IRF7 in the natural sample. Adriamycin showed high levels of IRF3, which was not confirmed in my RT-PCR data.

Genomatix Pathway	P-value
defense response	3.76E-13
immune response	4.22E-12
immune system process	6.55E-10
cytokine-mediated signaling pathway	6.76E-10
cellular response to cytokine stimulus	1.55E-09
response to stress	2.86E-09
inflammatory response	8.54E-09
response to cytokine stimulus	2.74E-08
innate immune response	3.65E-08
response to wounding	4.60E-07
response to organic substance	1.57E-06
cellular response to organic substance	2.20E-06
regulation of I-kappaB kinase/NF-kappaB cascade	3.72E-06
cell surface receptor linked signaling pathway	5.55E-06
response to stimulus	6.40E-06
response to molecule of bacterial origin	6.51E-06
positive regulation vascular endothelial growth factor production	7.55E-06
response to chemical stimulus	7.77E-06
multi-organism process	8.67E-06
regulation of interleukin-6 production	1.15E-05

Table 3: Genomatix Pathway Analysis of the 48 Senescence-Associated Genes. The 48 senescence-associated genes are involved with immune system pathways. The top 20 pathways represented by the 48 genes common to all types of senescence but not quiescence according to Genomatix analysis are shown here, sorted from lowest p-value to highest p-value. All pathways are statistically significant.

Ingenuity Pathway	P-value
Dendritic Cell Maturation	0.00525
Role of Cytokines in Mediating Communication between Immune Cells	0.00687
Atherosclerosis Signaling	0.00799
NF-κB Signaling	0.015
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	0.0194
Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	0.0231
Graft-versus-Host Disease Signaling	0.0262
IL-6 Signaling	0.0271
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	0.0287
LXR/RXR Activation	0.0311
TREM1 Signaling	0.0391
Hepatic Cholestasis	0.0391
IL-10 Signaling	0.0478
Acute Phase Response Signaling	0.049
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	0.0555
Communication between Innate and Adaptive Immune Cells	0.0572
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	0.062
PPAR Signaling	0.0639
FXR/RXR Activation	0.0665
IL-1 Signaling	0.0665

Table 4: Ingenuity Pathway Analysis of the 48 Senescence-Associated Genes. The 48 senescence-associated genes again are shown to be involved with immune system pathways. The top 20 pathways represented by these genes according to Ingenuity Pathway Analysis are shown here, sorted from lowest p-value to highest p-value. Fourteen pathways are statistically significant.

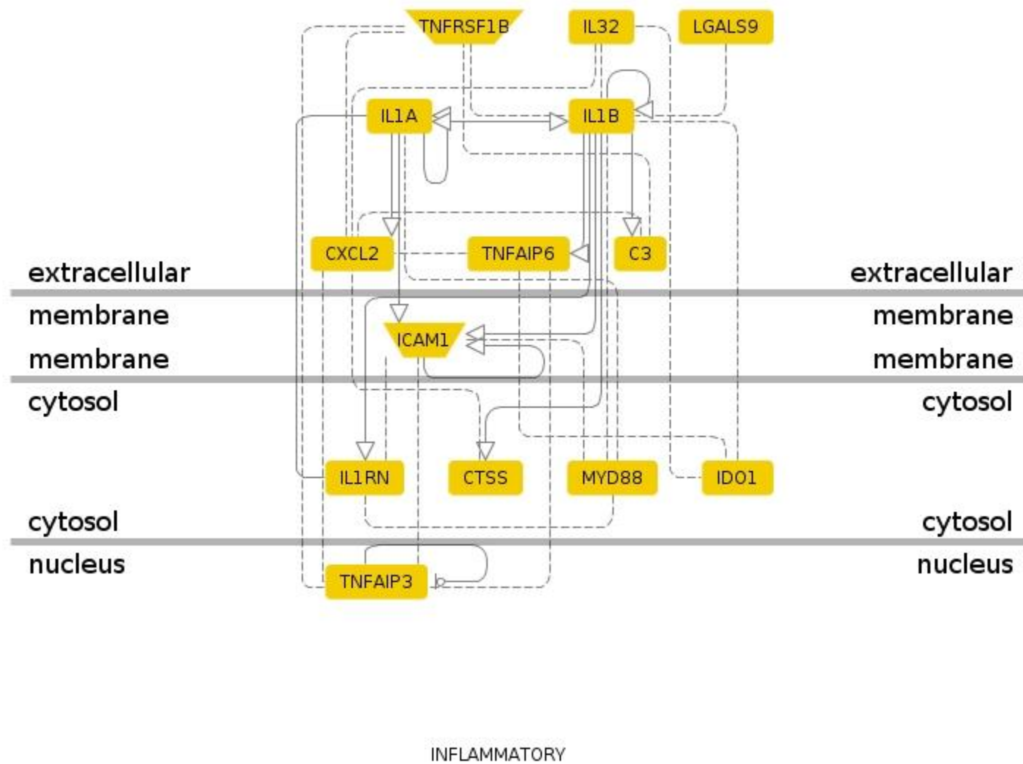


Figure 14: Inflammatory Pathway Activity According to RNA-seq Data. The inflammatory pathway was one of the most significant pathways to be important in all types of senescence compared to immortalization. Shown here is a network of the inflammatory pathway genes that were in all 4 types of senescence based on the RNA-seq data. Arrows indicate activation, and dashed lines indicate experimental validation. All of these genes were up-regulated in the 4 types of senescence compared to immortalization. Figure drawn using Genomatix.

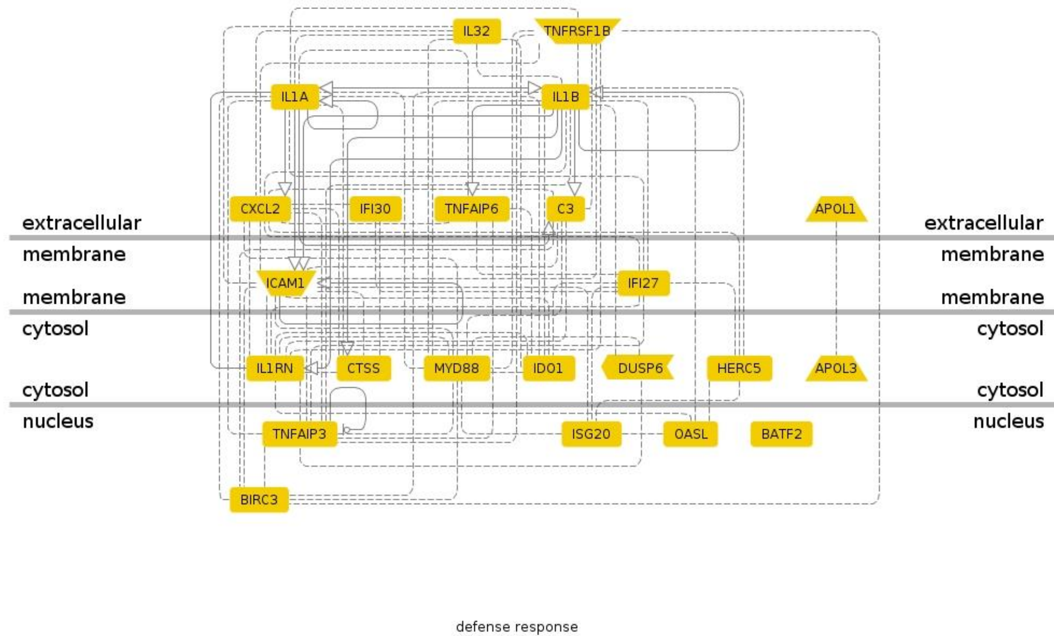


Figure 15: Defense Response Activity According to RNA-seq Data. Defense response was another highly significant pathway shown to be related to all 4 types of senescence. As shown above, the genes indicated in this pathway have a variety of complex interactions. Arrows indicate activation, while dashed lines indicate experimental validation. All of these genes were up-regulated in the 4 types of senescence compared to immortalization. Figure drawn using Genomatix.

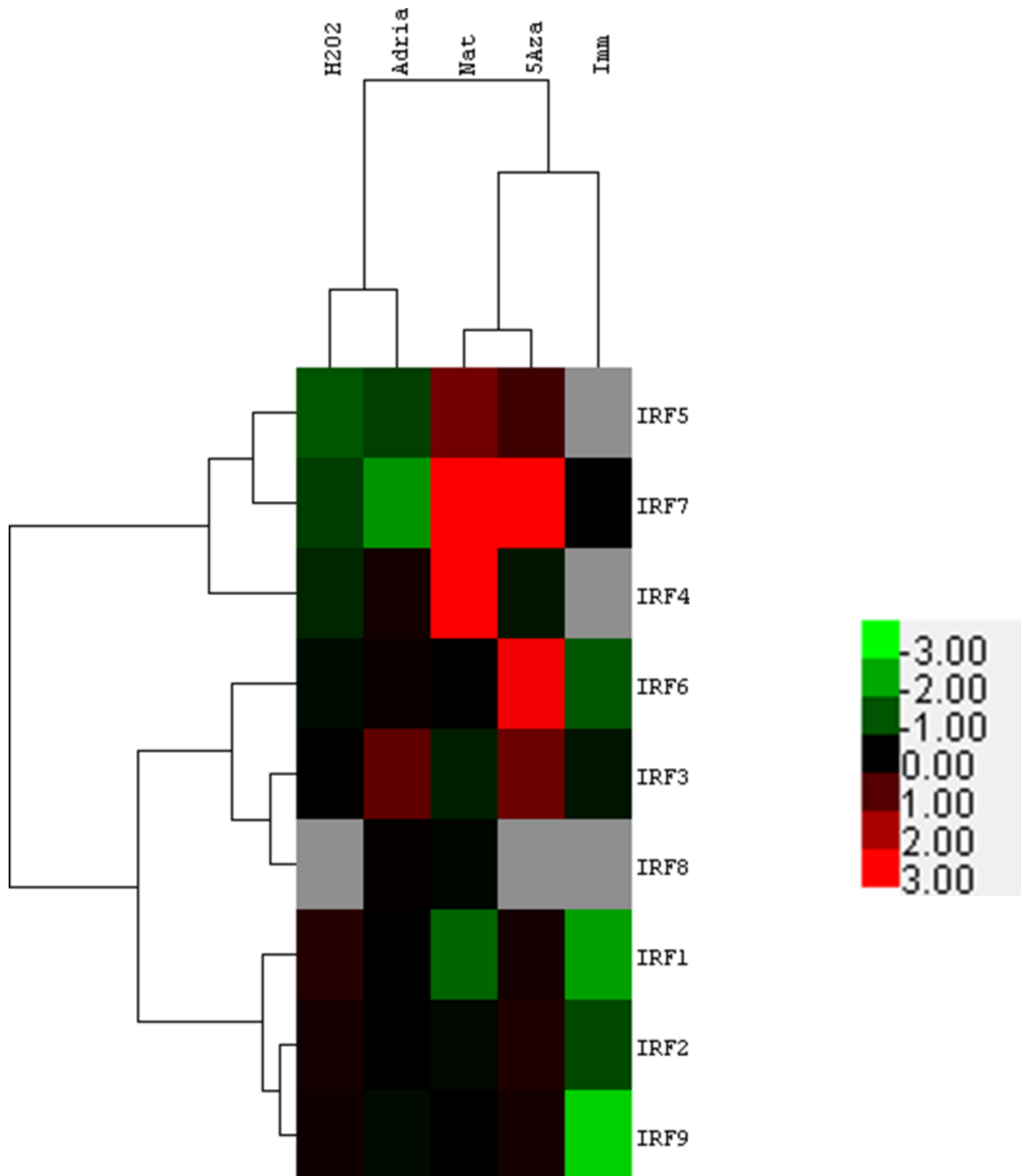


Figure 16: Cluster Analysis of Interferon Regulatory Factors in Senescence and Immortalization. IRF expression is variable among the different types of senescence, but as a whole, shows up-regulation in senescence compared to the immortal cells. Immortal, 5-aza, adria, and H_2O_2 samples are shown. Red bars indicate up-regulation and green bars indicate down-regulation compared to the median expression of a given gene.

3.4 RNA-seq Demonstrates the Significance of the IL1 Pathway in All Types of Senescence

After showing the interferon pathway's presence in all 4 types of senescence through Genomatix and IPA analysis based on the 48 genes in common in all types of senescence but not quiescence, a slightly different approach was used to identify pathways. Signalling Pathway Impact Analysis was performed by the WSU Applied Genomics Technology Center, specifically Dr. Calin Voichita performed the analysis under the supervision of Professor Sorin Draghici, Department of Computer Science, WSU. Dr. Voichita performed SPIA analysis on all genes differentially expressed in all types of cellular senescence. SPIA, or Signaling Pathway Impact Analysis, identifies significant pathways based on over-representation of differentially expressed genes in pathways, as well as abnormal changes in pathways measured by changes across pathway topology [79]. This type of analysis is different from Genomatix and Ingenuity, which rely on gene set enrichment analysis (GSEA). Based on the genes differentially expressed in the 4 types of senescence compared to immortalization, the top 9 pathways that were given all involved the interferon and immune system pathways that were identified previously (Table 5). Based on these 9 pathways, the genes that comprised these pathways were extracted and the genes present in all 4 types of senescence were considered for further study. This consisted of 9 genes that were interferon related and involved with cellular senescence (Tables 6 and 7). These 9 genes represent a subset of genes also identified in the previous identification of 48 genes that were considered senescence-associated. However IL8 (one of the 9 genes identified) was not included in the list of 48 genes because it was also differentially expressed in quiescence. However, upon further examination, this gene was

actually down-regulated in quiescence compared to immortalization, while being up-regulated during senescence, so while it was omitted from the original list of 48 senescence-associated genes, it is indeed senescence-associated.

Five out of 9 of the identified interferon-related genes were present in one pathway, which was the interleukin 1 pathway. These genes were shown to be up-regulated in senescence compared to immortalization in MDAH041 cells through RT-PCR, and IL1α and Il1β were shown to be up-regulated in 3 types of induced senescence compared to immortalization at a protein level in MDAH041 cells through immunocytochemistry. I performed additional confirmation of this pathway through RT-PCR of MDAH087-N and MDAH087-1 cells, which showed up-regulation of IL1α and IL1β in the 3 types of induced senescence compared to immortalization. The Il1 pathway was therefore confirmed to be up-regulated in 4 types of senescence compared to immortalization in multiple cell lines.

Cytokine-cytokine receptor interaction
Chemokine signaling pathway
TGF-beta signaling pathway
Jak-STAT signaling pathway
Toll-like receptor signaling pathway
Antigen processing and presentation
T cell receptor signaling pathway
B cell receptor signaling pathway
Natural killer cell mediated cytotoxicity

Table 5: Signaling Pathway Impact Analysis Reveals Inflammatory Pathways in all 4 Types of Senescence. Shown here are the 9 statistically significant pathways represented in at least one type of senescence as determined by SPIA. There were 209 genes that comprised these pathways. These genes were then further analyzed to identify the genes that were differentially expressed in all 4 types of senescence. This yielded a total of 9 genes that were considered for further study.

CTSS – cathepsin S
CXCL2 - chemokine
GNG11 – guanine nucleotide binding protein subunit gamma 11
ICAM1 – intercellular adhesion molecule
IFI30 – gamma IFN inducible lysosomal thiol reductase
IL1A – interleukin 1A
IL1B – interleukin 1B
IL8 – interleukin 8
MYD88 – myeloid differentiation primary response gene

Table 6: Nine Genes Differentially Expressed in all 4 Types of Senescence According to SPIA Analysis of Senescent Pathways. These genes are present in the 9 pathways listed in table 5 and are differentially expressed in all 4 types of senescence compared to immortalization. The genes shown in red are all members of the IL1 pathway and were chosen for further analysis.

	H_2O_2	Adria	Nat	5-aza
IL1a	5.82	3.44	5.98	8.87
IL1b	6.38	3.75	4.58	9.57
Myd88	1.92	1.32	1.26	1.62
ICAM1	3.95	2.94	3.73	7.23
IL8	5.55	3.10	2.78	7.28

Table 7: Gene Expression of the IL1 Pathway According to RNA-seq Data. Five members of the IL1 pathway were up-regulated in all 4 types of senescence compared to immortalization. Log_2 Fold changes in all four types of senescence for 5 of the 9 genes indicated by SPIA analysis to be important in senescence. These values represent differential expression analysis of the original RNA-seq data, with each condition normalized to the immortal control. All 5 of the genes were up-regulated compared to the immortal control.

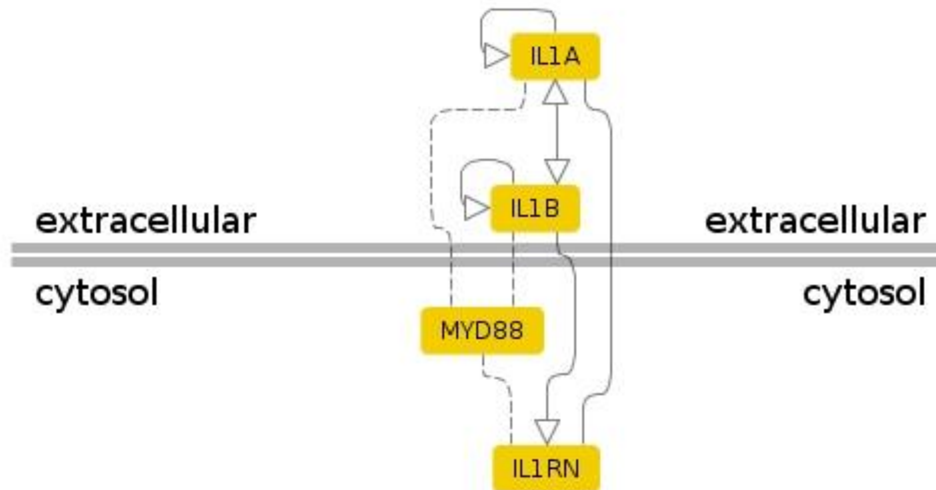
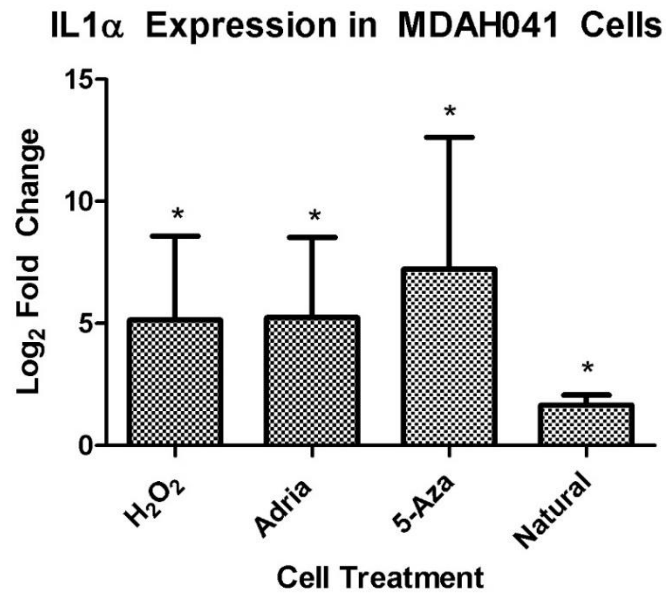


Figure 17: IL1 Pathway Activity According to RNA-Seq Data. The Interleukin 1 pathway was a significant pathway in all types of senescence according to the Genomatix Genome Analyzer. Arrows indicate activation, while dashed lines indicate experimental validation. IL1α, IL1β and IL1RN (IL1 receptor antagonist) all bind the IL1R (IL1 receptor), and then interact with the adapter protein Myd88. The IL1R did not show differential expression in our RNA-seq data.

3.5 Confirmation of the IL1 Pathway in All Types of Cellular Senescence

In order to validate the RNA-seq data, RT-PCR was performed to confirm the up-regulation of the Il1 pathway in all 4 types of cellular senescence. The up-regulation of IL1α, IL1β, Myd88, ICAM1 and IL8 were all confirmed in the MDAH041 cells (Figures 18 and 19), and IL1α and IL1β were confirmed in induced senescence of the MDAH087-1 and MDAH087-N cells (Figures 20 and 21). Naturally senescent samples were not obtained for MDAH087 because the cells progress from decreased growth into a state of crisis where they proliferate quickly, and therefore an accurately senescent sample was not attainable. In addition to RT-PCR, protein levels were also assessed using immunocytochemistry of MDAH041 cells. Natural senescence was not included in the protein analysis of the IL1 pathway because they could not be plated on chamber slides properly. If plated too early while the cells are not senescent, the cells will become too confluent for protein analysis, and if plated during senescence, the cells will not adhere to the plate. Additionally, naturally senescent MDAH087 cells were not included in RT-PCR analysis because of their difficulty to obtain. The MDAH087 cells typically stop proflieration in senescence very briefly, and enter crisis where they begin proliferating again, making isoluation of naturally senescent MDAH087 cells difficult.



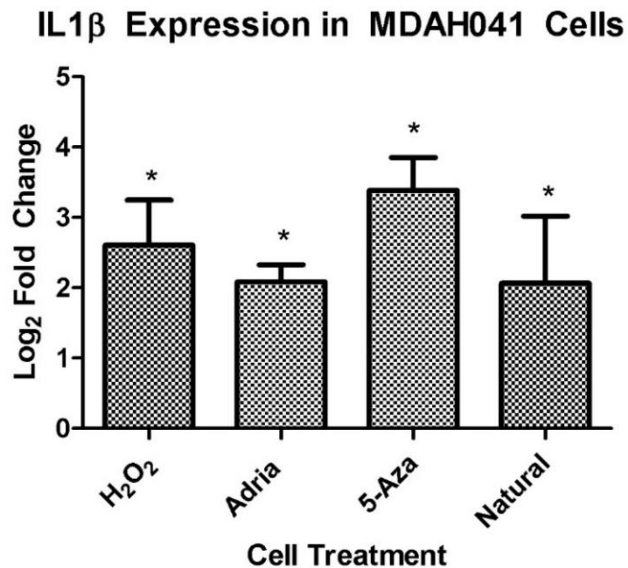
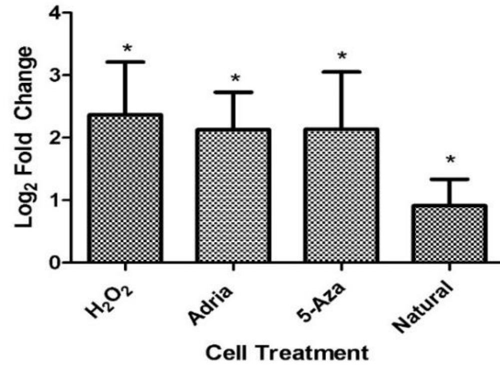
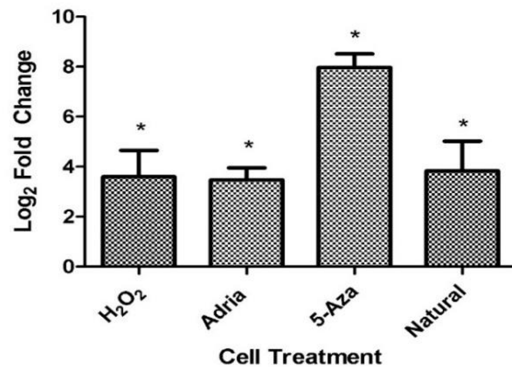


Figure 18: IL1α and Il1β Expression in MDAH041 Cells. IL1α and Il1β were both up-regulated in all 4 types of senescence compared to immortalization in the MDAH041 cells. Changes shown are RT-PCR Log_2 fold changes relative to immortalization. Error bars indicate standard deviation of 3 biological replicates andastericks indicate a significant difference from immortalization with $p<0.05$.





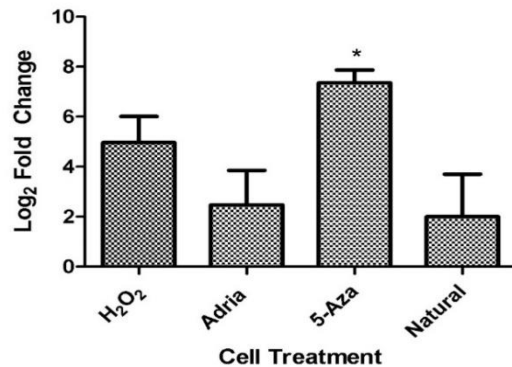
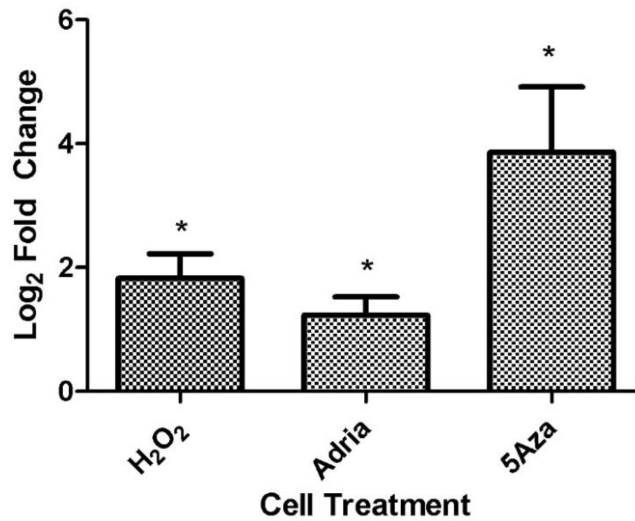


Figure 19: Myd88, ICAM1 and IL8 Expression in MDAH041 Cells. Myd88, ICAM1 and IL8 were all up-regulated in all 4 types of senescence compared to immortalization in the MDAH041 cells. Values shown are $\log_2$ fold changes in MDAH041 cells relative to immortalization. Error bars indicate standard deviation of 3 biological replicates and astericks indicate a significant difference from immortalization with $p<0.05$.



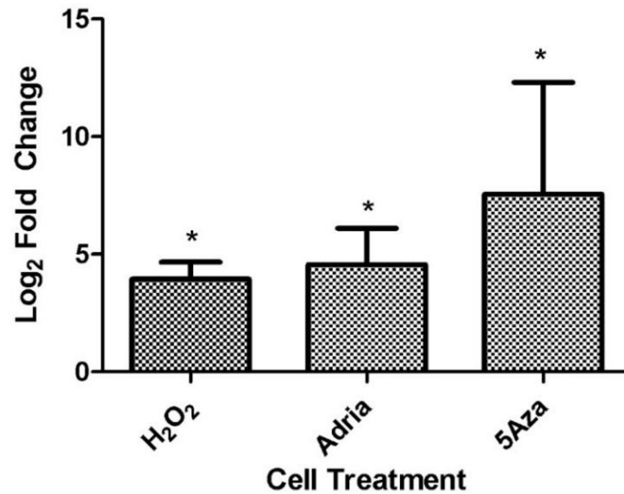
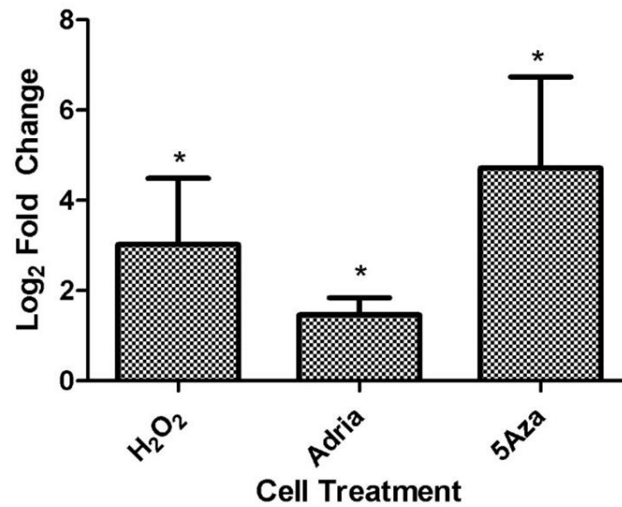


Figure 20: IL1α and Il1β Expression in MDAH087-1 Cells. IL1α and Il1β were up-regulated in the 3 types of induced senescence compared to immortalization in the 087-1 cells. Values shown are $\log_2$ fold changes compared to immortal MDAH087-1 cells. Error bars are indicative of standard deviation of 3 biological replicates and astericks indicate significant difference compared to immortalization with $p<0.05$.



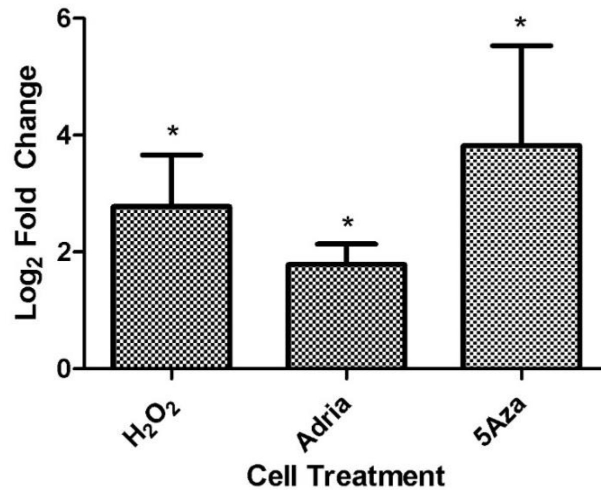


Figure 21: IL1α and IL1β Expression in MDAH087-N Cells. IL1α and Il1β were up-regulated in the 3 types of induced senescence compared to immortalization in the 087-N cells. Values shown are log_2 fold changes compared to immortal MDAH087-N cells. Error bars indicate standard deviation of 3 biological replicates and astericks represent a significant difference from immortalization with $p<0.05$.

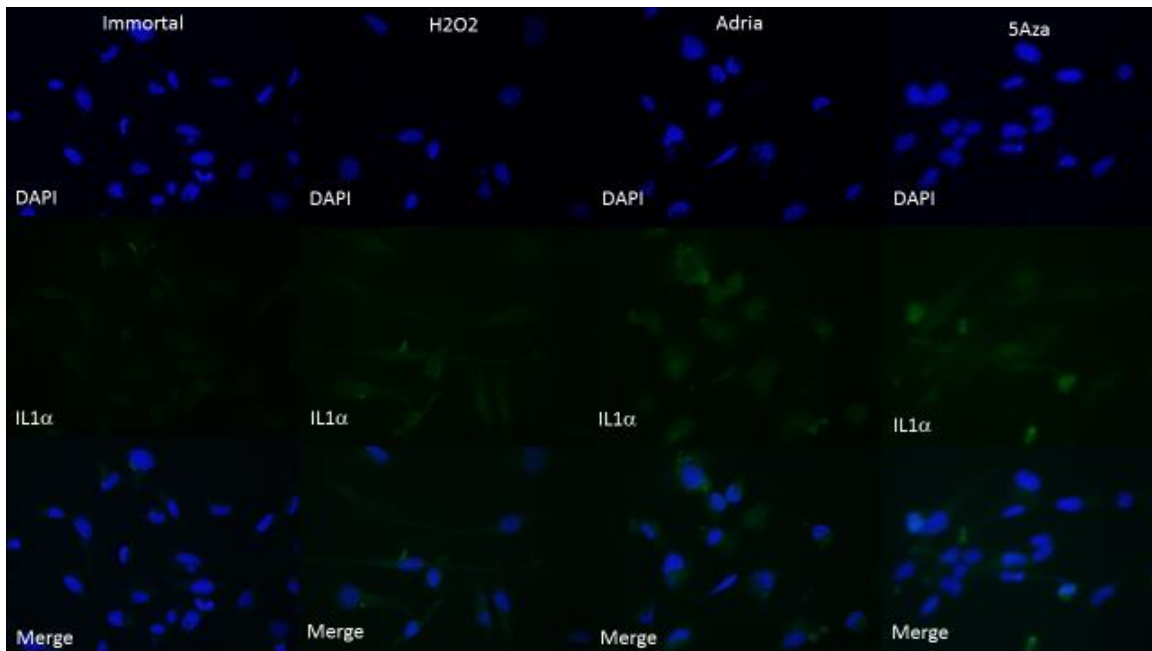


Figure 22: Immunocytochemistry Analysis of Il1α Protein Levels in MDAH041 Cells. IL1α was up-regulated at a protein level in the 3 types of induced senescence compared to immortalization. Blue staining indicates DAPI (top row), green staining indicates IL1α (middle row) and the bottom row shows a merged representation of both stainings.

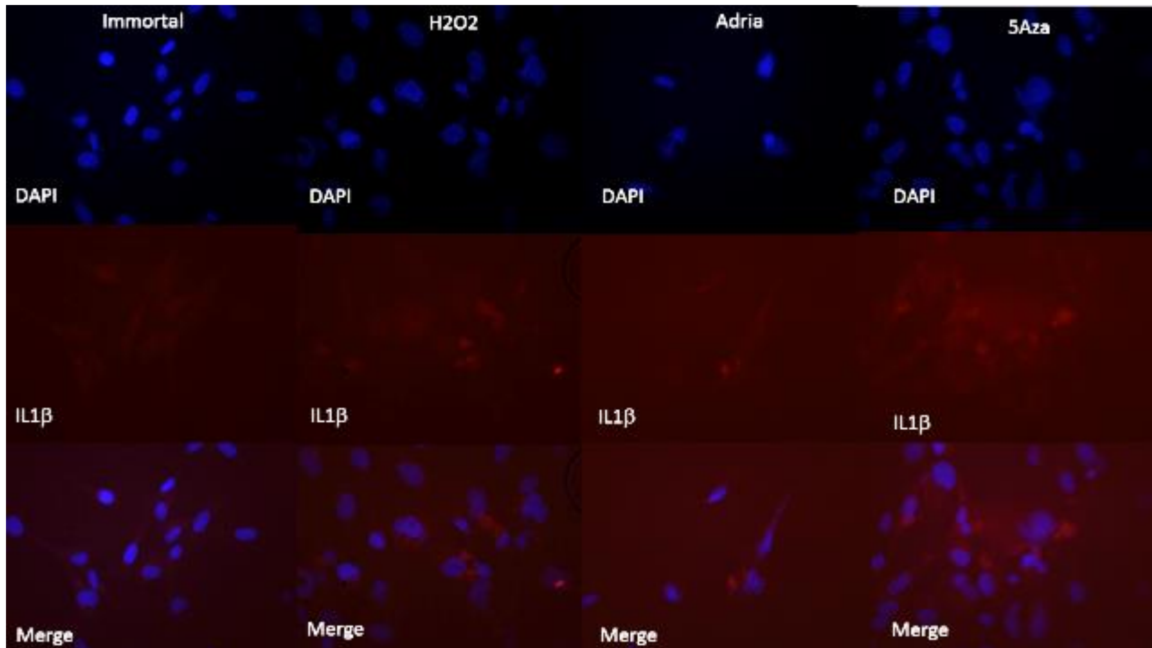


Figure 23: Immunocytochemistry Analysis of Il1β Protein Levels in MDAH041 Cells. IL1β is up-regulated at a protein level in the 3 types of induced senescence compared to immortalization. Blue staining indicates DAPI (top row), red staining indicates IL1β (middle row) and the bottom row shows a merged representation of both stainings.

3.6 Histone Modifications in Senescence

Signaling Pathway Impact Analysis, or SPIA was performed with the help of the Wayne State University Computer Science Department on the differentially expressed genes in invidivual types of senescence compared to immortalization in order to elucidate any pathways unique to each type of senescence. I chose to focus on adriamycin-induced senescence because of its relevance to cancer chemotherapy. Adriamycin-induced senescence yielded the histone modification or chromatin modification pathway in this specific type of senescence. Based on this analysis, a list of genes involved with chromatin modification were elucidated from the SPIA software, and a table of values was composed consisting of the different values of each gene (Table 8).

Clustering analysis of these genes was performed (Figure 24). The first column, which represents adriamycin-induced changes in gene expression, show a greater down-regulation of most genes than the other three types of senescence, which for the most part show only small levels of up-regulation or down-regulation of the genes related to adriamycin-induced senescence.

PRDM9 was analyzed, based on its high up-regulation in adriamycin-induced senescence. PRDM9 is a histone H3 methyltransferase, which contains a PR domain [81]. This protein is thought to dictate genetic recombination hotspots through its sequence-specific binding to zinc finger domains [82]. Specifically, PRDM9 trimethylates H3K4, which is typically a histone mark that indicates active transcription. Therefore, PRDM9 was an interesting candidate for regulation of senescence because up-regulation of an active histone mark during senescence could cause increased expression of senescence genes. PRDM9 was confirmed to be upregulated in adriamycin-induced senescence

compared to the immortal control, however it was also upregulated in 5-aza-induced senescence and H_2O_2-induced senescence (Figure 25). It appeared to be down-regulated in natural senescence. This may represent a unique method of induction of senescence in adriamycin treated cells.

Additionally, SUV420H1 was analyzed based on its down-regulation in adriamycin-induced senescence. This gene was chosen based on having one of the highest down-regulations of the chromatin modification genes, as well as its known activity as a methyltransferase. SUV420H1 is a histone H4K20 methyltransferase and contains a SET domain which allows for interactions with other proteins [83]. Specifically, SUV420H1 trimethylates H4K20, which is typically a repressive histone mark. Therefore, SUV420H1 was an interesting candidate for the regulation of senescence bceause down-regulation of a respressive histone mark during senescence could cause increased expression of a given gene during cellular senescence. It was confirmed to be down-regulated in adriamycin-induced senescence, however it was also downregulated in H_2O_2-induced senescence, 5-aza induced senescence, and natural senescence (Figure 26). Based on the large amount of chromatin modifying genes differentially expressed in adriamycin-induced senescence, as well as the confirmation of two genes, it is probable that adriamycin-induced senescence involves chromatin remodeling, which is a novel method of induction of senescence for this agent, and is similar to the mechanism of 5-aza-induced senescence. 5-aza-induced senescence is essentially an epigenetic reversal of immortalization, as 5-aza is an inhibitor of DNA methyltransferase, and was shown to cause re-activation of several interferon genes upon treatment of immortal cells [49].

Gene Name	Log_2 Fold Change	Gene Function
TADA2A	-1.46	Histone acetylation, activation
AEBP2	-1.24	Histone methylation, repression
SUV420H1	-1.18	Histone methylation, repression
SMARCA1	-1.15	Helicase/ATPase activity, activation
NSD1	-1.14	Histone methylation, repression/activation
HLTF	-1.12	Helicase/ATPase activity, repression/activation
NIPBL	-1.06	Chromatin structure
MLL5	-1.02	Histone methylation, activation
MORF4L1	-1.02	Histone acetylation, activation
RNF2	-1.02	Histone ubiquitination, repression
USP16	-0.99	Histone de-ubiquitination, activation
SUDS3	-0.96	Histone de-acetylation, repression
HUWE1	-0.93	Histone ubiquitination, repression
PBRM1	-0.92	Nucleosome remodeling, activation/repression
SMARCE1	-0.86	Nucleosome remodeling, activation/repression
PHF15	1.02	Histone acetylation, activation
CPA4	2.45	Histone acetylation, activation
PRDM9	4.83	Histone methylation, activation

Table 8: Chromatin Modification Genes Differentially Expressed in adriamycin-induced Senescence. Log_2 fold change determined by RNA-seq for each chromatin modifying gene as well as probable functions are listed. This chromatin modification of adriamycin-induced senescence could represent a novel mechanism of induction of senescence for adriamycin.

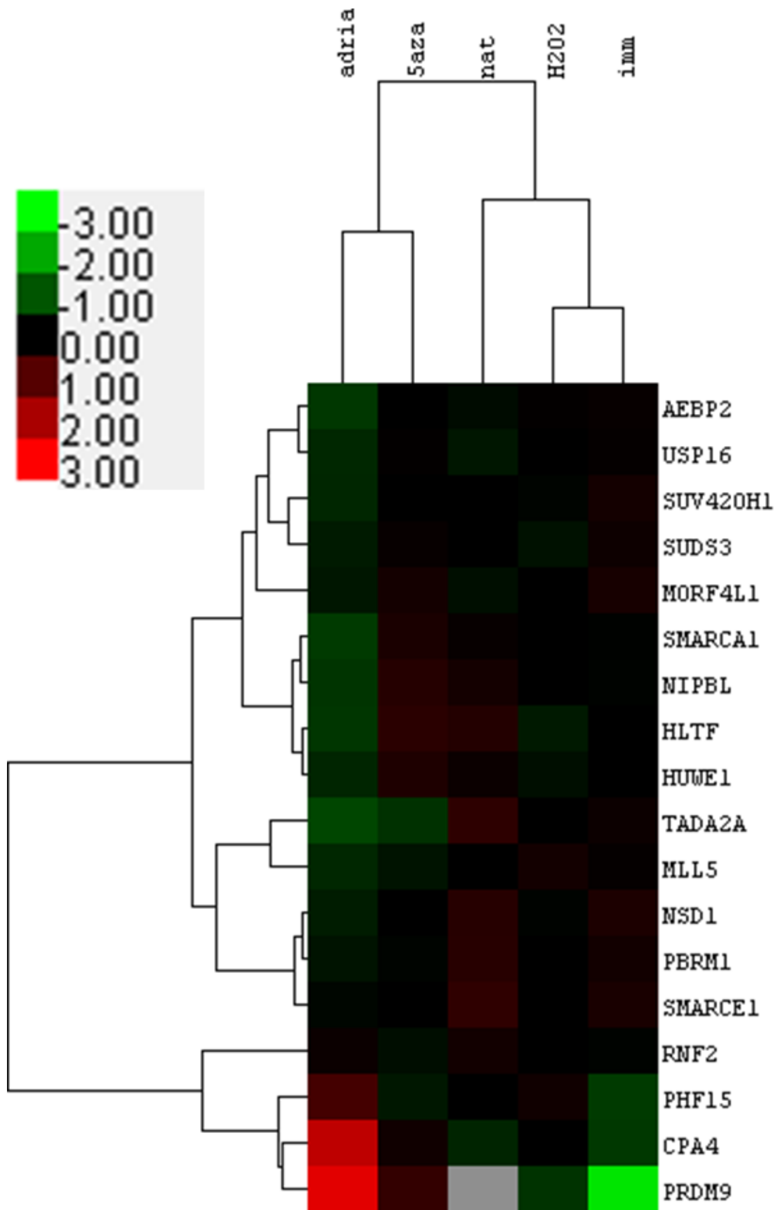


Figure 24: Clustering Analysis of the Genes Shown to be Unique to adriamcyin-induced senescence. Adriamycin-induced senescence shows a slight down-regulation of most chromatin modifying genes compared to the other 3 types of senescence. Green bars indicate relative down-regulation and red bars indicate relative up-regulation compared to median expression for a given gene.

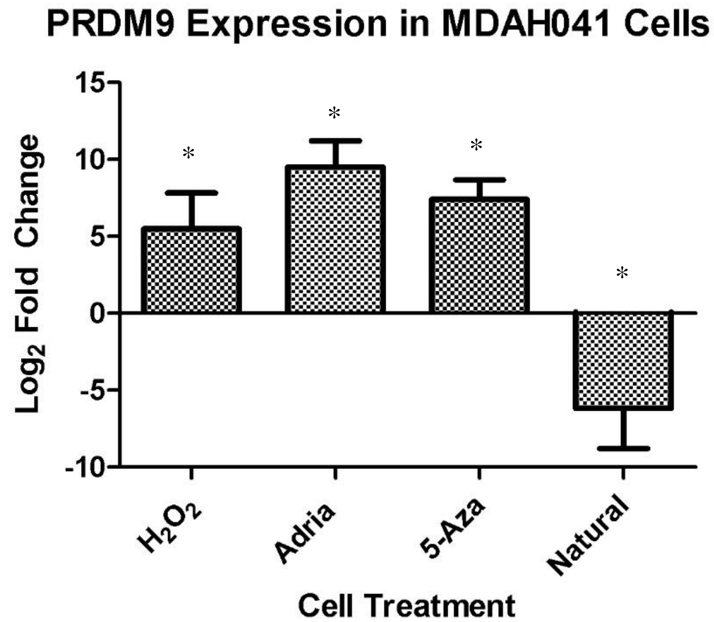


Figure 25: PRDM9 Expression in MDAH041 Cells. PRDM9 was up-regulated in the 3 types of induced senescence compared to immortalization, but down-regulated in natural senescence compared to immortalization. Changes shown are log_2 fold changes of PRDM9 relative to immortalization. Error bars indicate standard deviation of 3 biological replicates. Astericks indicate statistically different changes from the immortal control where $p<0.05$.

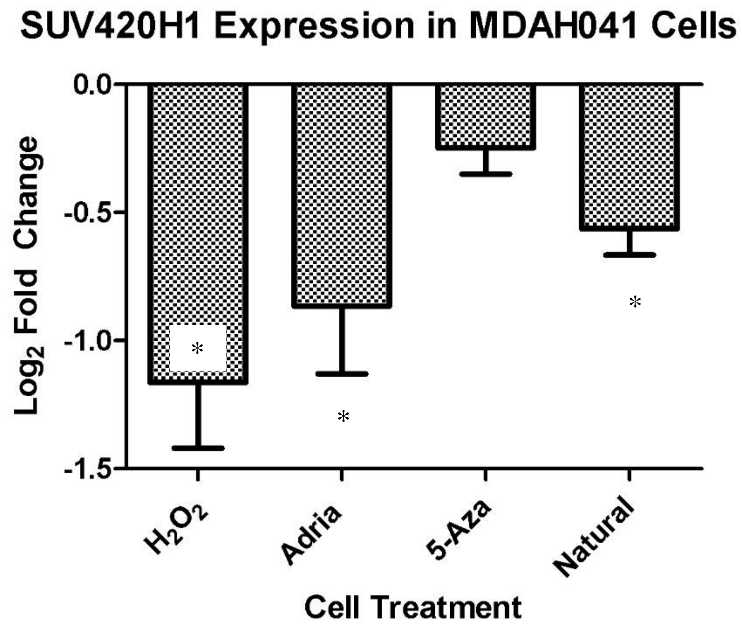


Figure 26: SUV420H1 Expression in MDAH041 Cells. SUV420H1 is down-regulated in all 4 types of senescence compared to immortalization. Changes shown are log_2 fold changes relative to immortalization. Error bars indicate standard deviation of 3 biological replicates. Astericks indicate stastistically significant changes from immortalization with $p<0.05$.

3.7 Senescence Associated Secretory Phenotype Profiles

The senescence associated secretory phenotype is a group of secreted factors released from senescent cells into the microenvironment. The RNA-seq data was analyzed for the presence of all known SASP factors [84]. These factors are grouped into three categories by their regulation: High increase (4+ fold change), intermediate increase (2-4 fold change) and small increase (below 2 fold change) based on previous studies establishing these changes. In order to assess the differences in the senescence associated secretory phenotype profiles among the different types of senescence, as well as the immortal control, clustering analysis was performed of the genes that make up the pathway. Analysis was performed using each category (high increase, intermediate increase and low increase) separately, and with all the categories of genes together to see overall changes.

Clustering analysis of all genes showed immortalization to have a high number of downregulated genes, whereas the multiple types of senescence show an increase in several of the genes (Figure 27). Firstly, this confirms the senescent phenotype of the senescent cells, especially when compared to the immortal control which shows downregulation of these genes. Secondly, this clustering analysis shows that again the naturally senescent cells group most closely with the 5-aza-treated senescent cells, showing that 5-aza-induced senescence mimics the natural aging process of DNA demethylation. Aging cells have been shown to have decreased amounts of methylation with age, whereas immortal cells maintain methylation [85]. Therefore, the 5-aza induced senescence resembles natural senescence.

Clustering analysis of SASP components with high fold changes was performed (Figure 28). Accordingly, the immortal control shows downregulation of every gene listed

(with exception of two which no values were found) and the senescent samples show upregulation of these SASP genes. 5-aza showed the highest number of upregulated genes, indicating a very strong SASP response, whereas adriamycin treatment didn't show as much of the SASP as expected, given that DNA damage is known to cause a SASP response.

Clustering analysis of the SASP components with intermediate fold change revealed that again the immortal control has several of these genes downregulated, and the senescent samples have them upregulated (Figure 29). 5-aza again shows the highest amount of upregulation, indicating a strong SASP response. Adriamycin treated cells again don't show a high amount of upregulation as expected, given that DNA damage is a known cause of SASP [41]. There were 5 genes from this group of SASP members that were not found in our dataset (gray boxes).

Lastly, clustering analysis of SASP components with small fold changes was performed (Figure 30). There were a large number of genes in this group that were not found in our dataset (gray boxes). The immortal control again shows downregulation of most of the genes that were found in the dataset, while the senescent samples show upregulation of these genes. Again, the 5-aza treated sample shows the highest amount of upregulation and a therefore a strong senescent response. Adriamycin shows a large number that were upregulated, but also a fair amount that were downregulated. This is somewhat unexpected as DNA damage is known to cause a SASP response.

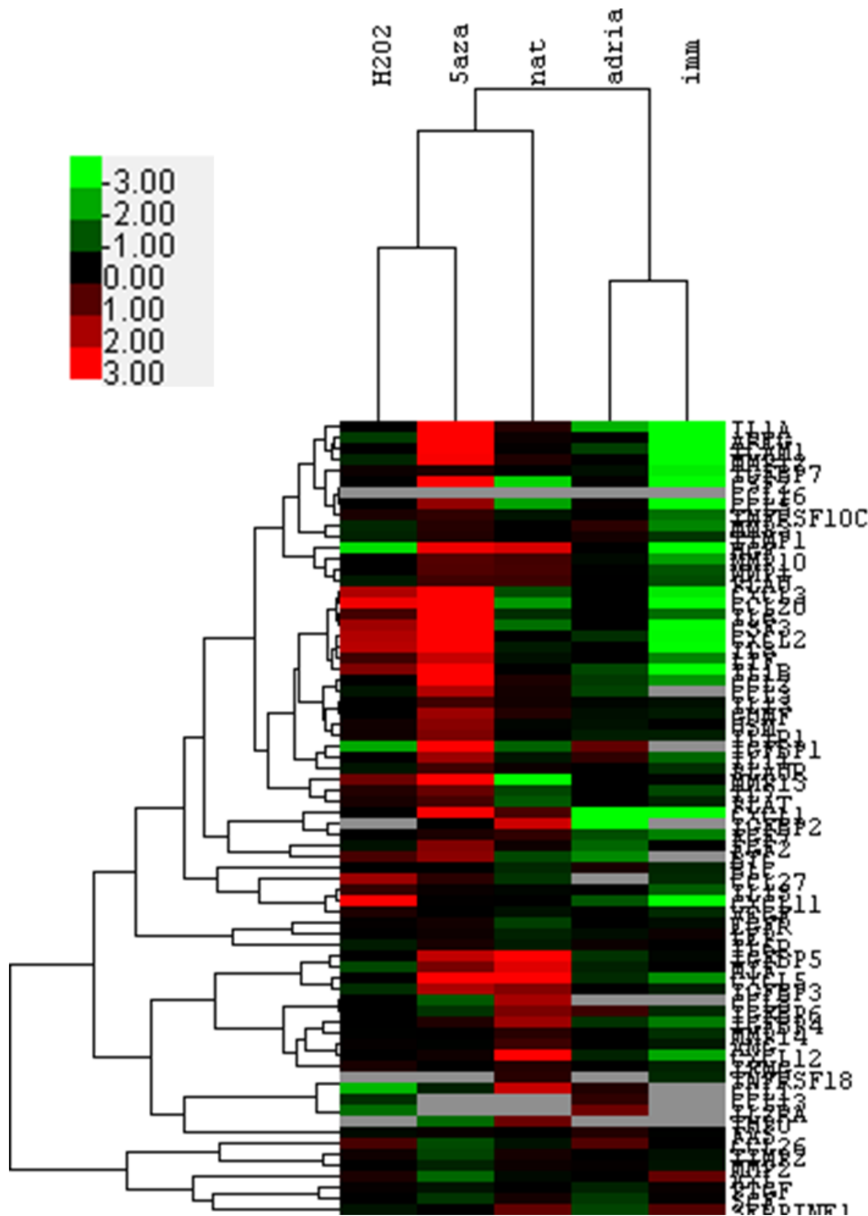


Figure 27: Clustering Analysis of all Known SASP Components. All known senescence associated secretory phenotype genes in all types of senescence and immortalization. Empty gray boxes indicate that the gene was not found in the given sample according to RNA-seq. 12 genes were not shown based on not being present in RNA-seq data for any of the samples. They were: GCP2, TNFRSF18, NAP2, OPG, SPG130, ACRP30, BLC, CCL16, MSP-a, SERPINEB2, SCF, and VEGF.

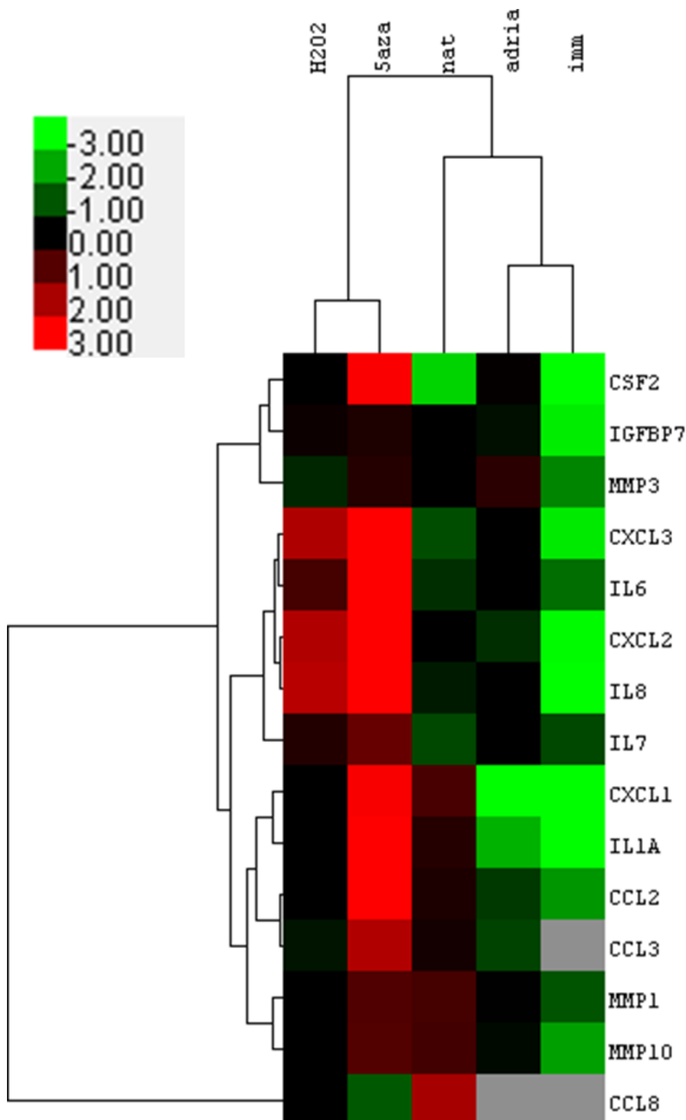


Figure 28: Clustering Analysis of SASP Components with High Fold Changes. Senescent associated secretory phenotype genes with typically high fold changes, as described by Freund et al. [84]. Gray boxes indicate where a value was not found for a given gene in our dataset. Red boxes indicate relative up-regulation while green boxes indicate relative down-regulation compared to the median expression for a given gene.

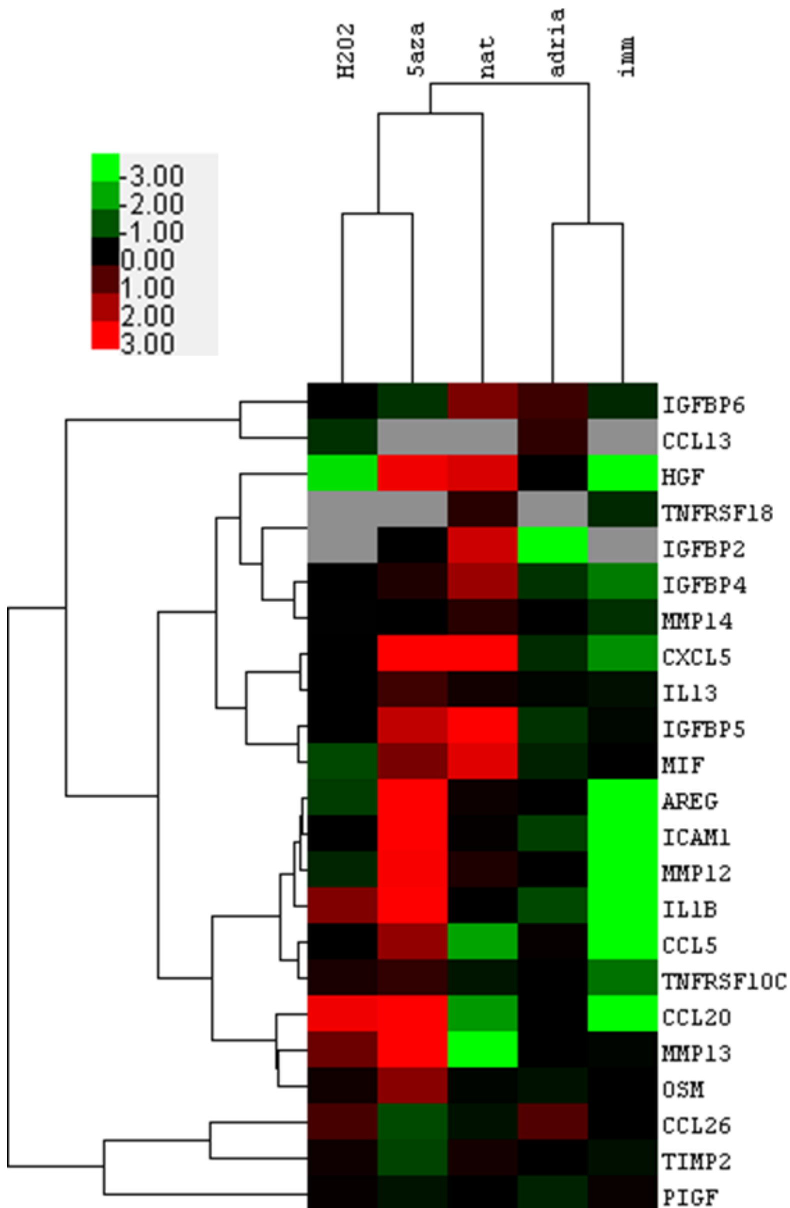


Figure 29: Clustering Analysis of SASP Components with Intermediate Fold Change. Senescent associated secretory phenotype genes with an intermediate fold change as described by Freund at al [84]. Gray boxes indicate that no value was found in our data for the given gene. Red boxes indicate relative up-regulation while green boxes indicate relative down-regulation compared to the median expression for a given gene.

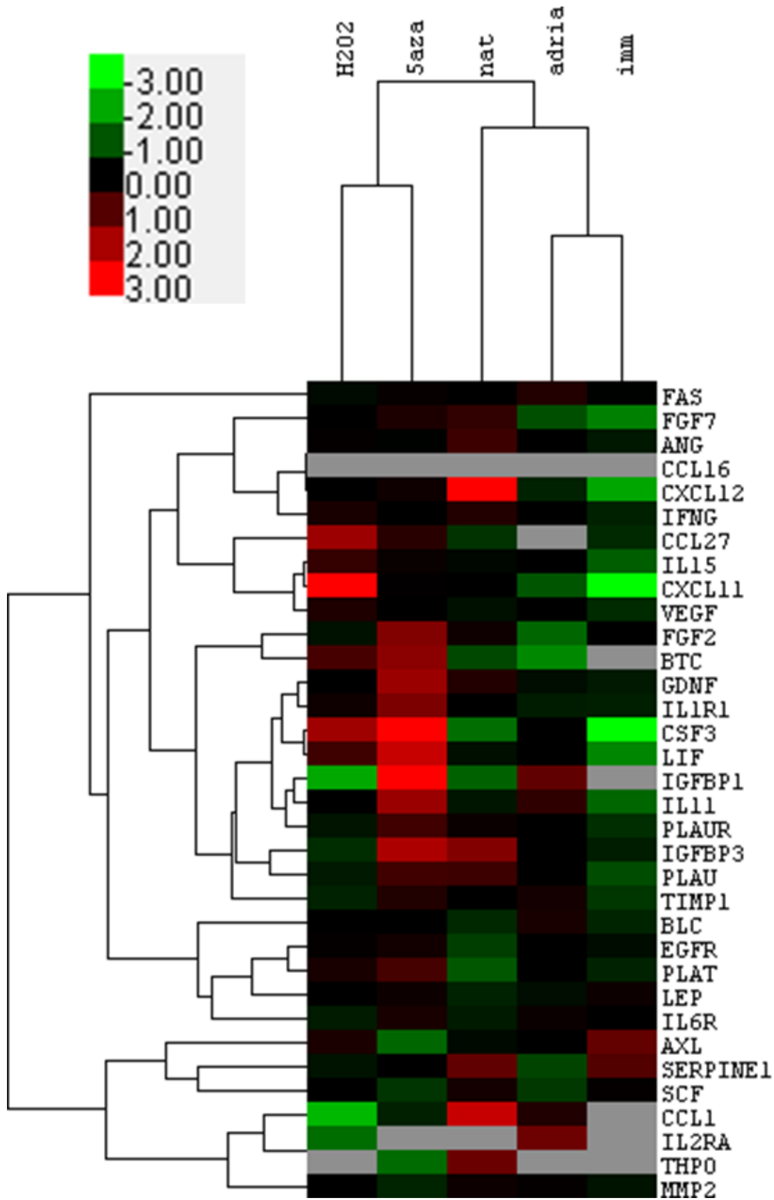


Figure 30: Clustering Analysis of SASP Components with Small Fold Change. Senescent associated secretory phenotype genes with a small upregulation according to Freund et al. [84]. Gray boxes indicate that no value was found in our data. Red boxes indicate relative up-regulation while green boxes indicate relative down-regulation compared to the median expression for a given gene.

CHAPTER 4

DISCUSSION

4.1 Goal of Study

Cellular senescence during aging is believed to be a tumor suppressive mechanism, through limiting the replication of a cell and thereby preventing acquisition of hazardous mutations [86]. Senescence can protect the cell from replicating once an oncogene is activated, again serving as a protective mechanism [30]. However, senescence also occurs in response to other stressors, such as chemotherapeutic agents and oxidative stress, which can hinder therapeutic response [52]. Given the various mechanisms that generate senescent cells, it was important to understand these different types of senescence and compare their pathways by gene expression profiling. Previous gene expression studies of cellular senescence have typically focused on a single mechanism of senescence, such as oncogene-induced senescence or senescence induced by radiation. In this thesis research, I utilized a cellular model that allowed comparison of multiple types of senescence within one cell line, allowing me to contrast natural or replicative senescence and induced senescence, as well as compare senescence and immortalization without confounding issues of genetic variation among cell lines.

4.2 The Role of the Inflammation and SASP in Senescence

Further complicating the understanding of senescence is the senescence-associated secretory phenotype, which involves a set of more than 70 secreted factors from most senescent cells that can cause inflammation, bystander senescence, and even transformation of surrounding pre-malignant cells [70]. Previously in the Tainsky laboratory, gene expression profiling was used to study epigenetic regulation of gene

expression during immortalization by comparison to 5-aza-2'-deoxycytidine-induced senescence. Those studies showed dysregulation of the cytoskeletal pathway, cell cycle pathway, and interferon pathway [22]. While the cytoskeletal pathway and cell cycle pathway gene expression changes were expected due to changes in cell morphology and withdrawal from the cell cycle during senescence, the interferon pathway was an unexpected finding. This role for the interferon pathway during cellular senescence that was identified by our lab has been cited as early evidence for presence of the senescence-associated secretory phenotype [41]. RNA-seq analysis of 4 types of senescence in the present thesis research has shown that up-regulation of the interferon pathway is present in all 4 types of senescence, and therefore it is likely that the senescence-associated secretory phenotype is also present in all types of senescence. Clustering analysis revealed that each type of senescence had at least some SASP factors up-regulated, although the exact SASP profiles were not consistent between samples. For example, 5-aza-induced senescence, natural senescence, and H_2O_2-induced senescence all had large numbers of genes up-regulated compared to immortal cells, but not the same genes. This could indicate that while various types of senescence utilize the same mechanisms and characteristics (such as SASP), the specific genes involved in these processes may vary.

Since the Tainsky study was published in 2003 showing a role for the interferon pathway in 5-aza-induced senescence, others have shown results consistent with those findings that interferon-related genes are up-regulated during senescence, but the reason for this is still largely unknown, with the exception of the hypothesis that the senescence-associated secretory phenotype relies on these gene expression changes [60, 64, 66, 69, 87]. Our lab has since shown various transcriptional regulators of the

interferon/inflammatory pathway can cause senescence when exogenously expressed, specifically IRF5 and IRF7 [45]. This thesis, utilizing RNA-seq analysis of multiple types of senescence, provides evidence that the interferon/inflammatory pathway is involved in all types of senescence studied (Natural, 5-aza, adriamycin, and H_2O_2), through pathway analysis, clustering analysis, and RT-PCR validation of gene expression. The pathway analysis of senescence-associated genes was consistent among 3 different software approaches (Tables 3, 4, 5), which reinforces the finding that the inflammatory pathway is important in all types of senescence, and not software-dependent. Clustering analysis of senescence-associated interferon genes, including the IRFs, showed that again the pathway itself is up-regulated but the same genes are not always up-regulated from mechanism to mechanism, indicating a universally necessary pathway with variable gene involvement.

In conjunction with pathway analysis of the 48 genes in common in all 4 types of senescence, which indicated the interferon/inflammatory pathway, I also compared our data to a list of known senescence-associated secretory phenotype genes to assess the level of secreted factors in each type of senescence. There were a large number of genes from this list present in our data, as expected (Figures 27, 28, 29, 30). Interestingly, the 5-aza-induced senescence produced a senescence-associated secretory phenotype that was more robust according to cluster analysis of gene expression comparing senescent cells to immortal cells. The 5-aza sample showed both an increased number of genes from the known set as well as increased levels of expression of genes compared to the other senescent samples. This wasn't necessarily surprising, given that the 5-aza-induced senescence sample showed the most robust fold changes in gene expression as well compared to other types of senescence when normalized to immortal cells. The 5-aza-

induced senescence also exhibited a greater number of genes that were differentially expressed compared to immortalization than the other types of senescence. When the senescence-associated secretory phenotype genes were analyzed using clustering software, the 5-aza-induced senescence associated most closely with natural senescence (Figures 27, 28, 29, 30).

This is consistent with the fact that 5-aza works through DNA demethylation and natural senescence is due, at least in part, by a natural and progressive demethylation of DNA as the cell ages [85]. When the 48 genes differentially expressed in all types of senescence were clustered, 5-aza-induced senescence clustered closely to natural senescence as well. It is also important to note that 5-aza- induced senescence reverses immortalization; genes that are up-regulated during 5-aza-induced senescence are generally silenced during immortalization and the interferon/inflammatory pathway is a good example of this. This is consistent with a recent study showing that 5-aza-induced senescence of chronic myeloid leukemia was a result of shortened telomeres, which is the mechanism by which natural, replicative senescence occurs [88]. This reinforces the concept that replicative senescence is mechanistically similar to 5-aza-induced senescence, and that 5-aza-induced senescence is the epigenetic reciprocal of immortalization and associated with stabilized telomeres. Previous studies have shown that cells can have telomere attrition and senescence in response to oncogenes [89], and this may contribute to tumor suppression. However, when hTERT is expressed in human cell lines in order to stabilize telomeres, the cell will still undergo induced senescence in response to irradiation or H_2O_2, independent of telomere length [90]. Consistently, MDAH041 immortal cells used in our study, which utilize telomerase to stabilize telomeres, were still able to undergo

senescence in response to H_2O_2 and adriamycin. This suggests that although replicative senescence and 5-aza-induced senescence may both involve telomere attrition, and adriamycin-induced and H_2O_2-induced do not involve telomere attrition, there is still a common pathway between all 4 types of senescence: the interferon pathway. This study provides evidence that although the mechanisms of each type of senescence are different, they involve common pathways.

The adriamycin-induced senescence provided the most intense and consistent senescence-associated β-galactosidase staining. However, the gene expression changes generally were not as robust as the 5-aza-induced senescence in terms of fold change. Additionally, it was interesting that the adriamycin-induced senescence did not have a strong senescence-associated secretory phenotype (the adriamycin-induced sample did not show high up-regulation of genes present in the SASP, Figure 27), given the strong senescence-associated β-galactosidase response and consistent with the fact that DNA damage is a known contributor to the secretory phenotype [41]. It will be interesting in future studies to determine whether the difference in secreted cytokines affects the amount of bystander senescence or transformation of surrounding cells.

4.3 The Role of the IL1 Pathway in Senescence

Among the several interferon/inflammatory genes that were found to be up-regulated, several of the genes were present within one pathway: the Interleukin 1 Pathway. Il1α, Il1β, Myd88, ICAM1 and IL8 were all confirmed through RT-PCR analysis to be up-regulated in the 4 types of senescence compared to immortal cells (Figures 17-23). It is interesting to note that Il1α, Il1β and IL8 are all canonical members of the senescence-associated secretory phenotype, indicating an association between increased gene

expression of these members and increased secretion. Paradoxically, the Interleukin 1 Receptor Antagonist was also up-regulated in all 4 types of senescence. However, the antagonist serves to inhibit binding of Il1α and Il1β to the Il1 receptor, and Il1α/Il1β have been shown to exhibit increased activity during senescence, so it is unlikely that the receptor antagonist is causing inhibition of the IL1 pathway [68].

The up-regulation of IL1 family members has been previously shown in various cell lines and senescence-inducing drugs. Bleomycin treatment of the Human sigmoid colon adenocarcinoma cell line HCA2 yields an up-regulation of IL1α and Il1β [68]. Inhibition of IL1α in that study decreased secretion of IL6 and IL8, which are major components of the SASP. Several cancer cell lines such as Hela, A549 and U2OS show up-regulated IL1 pathway members in response to 5-bromo-2'-deoxyuridine, distamycin, aphidicolin and hydroxyurea which all induce senescence [60]. BJ fibroblasts were shown to up-regulate IL1 during replicative senescence, oncogene-induced senescence and distamycin-induced senescence [69]. Conditioned media from those senescent cells was capable of inducing bystander senescence in BJ fibroblasts, and those cells also exhibited up-regulated IL1 expression. IMR90 cells have up-regulated IL1 pathway members, and knockdown of the IL1R which prevents binding of IL1α and Il1β causes a decrease in associated paracrine senescence [70]. Therefore, the up-regulation of IL1 pathway members that was observed in our RNA-seq data is consistent with other studies, but provides a better comparison of multiple types of senescence, as well as immortalization, within a single genetic background.

4.4 Quiescent Gene Expression

For the analysis of gene expression data, we included RNA from quiescent cells, in order to identify the portion of genes that were not senescence-specific but rather cell cycle specific, given that quiescent cells have withdrawn from the cell cycle albeit not permanently. I observed up-regulated 45 genes in all 4 types of senescence and quiescence, and therefore these 45 genes were not used for pathway analysis of senescence. Clustering analysis of these 45 genes showed that while the quiescent gene expression had significant overlap with the senescent gene expression, the quiescent sample associated very closely with the immortal proliferating sample in gene clustering analysis, possibly indicating the quiescent-associated genes in the quiescent sample were not as similar to senescent samples as they appear based only on fold change analysis. This further validated our reasoning for segregating these genes from the pathway analysis of senescence (Figure 9). Surprisingly, pathway analysis on the 45 genes showed interferon/inflammatory pathway as the most significant pathway (data not shown). Among these genes, there were several interferon induced proteins, including IFIT1, IFIT2, IFIT3, IFITM1, IFI44L, IFIH1, and IFI6. Additionally, IRF9, an interferon regulatory factor, was also up-regulated in the quiescent control, indicating a role for the IRFs in activating the interferon/inflammatory pathway in both senescence and quiescence. IRF3, IRF5 and IRF7 were found in the RNA-seq data to be up-regulated in senescence but were not consistent among the different types of senescence (Figures 5, 6, 7, 16).

The cell cycle kinase inhibitor p21, which promotes cell cycle arrest, was also up-regulated in the quiescent control compared to the immortal cells in the Cufflinks analysis of differential expression. However, when relative abundances of quiescent cell gene

expression were compared to the 4 types of senescent cells via gene clustering, p21 appeared slightly down-regulated compared to the senescent samples. Up-regulation of p21 would not be surprising given the fact that the quiescent samples are withdrawn from the cell cycle, and therefore p21 may be playing a role in cell cycle arrest. However, the fact that the quiescent sample showed up-regulation in the fold change analysis but down-regulation of p21 in clustering analysis indicates that the expression of p21 is not as robust in the quiescent samples as the senescent samples which are permanently withdrawn from the cell cycle.

4.5 Progressive Up-Regulation of Genes during Cellular Aging

In addition to comparing various types of senescence to elucidate common senescent pathways, we also wanted to analyze the progression of young, proliferating cells into naturally senescent cells. For this reason, we included lowest available passage cells (PD 10-12), low passage cells (PD 17-19), and naturally senescent cells (PD 28-30). Clustering analysis of the 48 senescence-associated genes revealed a relative down-regulation of these genes in the lowest passage sample, and a slight up-regulation of these genes in the low passage sample. This is likely to represent a property of cells proliferating at a slower rate and approaching senescence. When the cells stopped proliferating and entered natural senescence, the 48 genes were highly up-regulated. This illustrates an interesting progressive up-regulation of senescence-associated genes which is consistent with the theory that senescence genes are dominant and therefore up-regulated during senescence [91]. These senescence-associated genes were predominantly part of the inflammatory pathway indicating that the inflammatory/immune response increases as a cell ages and this response is silenced during immortalization. A recent RNA expression study utilized young, middle aged, old aged, and naturally senescent IMR90 cells to assess the progression of senescence-associated genes. A progressive down-regulation of 1149 genes was observed, which

corresponded to proliferation and replication pathways [11]. There was also a progressive up-regulation of 454 genes, including the senescence-associated p21 and p16; however pathway analysis of these 454 genes did not yield consistent pathway involvement. These data are consistent with our RNA-seq study, showing that several genes become increasingly up-regulated as a cell ages, concurrent with the cell proliferation decreasing with age and consequently showing a decrease in replication-related genes. The progressive up-regulation and down-regulation of genes is most likely due to the progressive loss of telomeres as a cell ages, as expression of hTERT in the IMR90 cells reverted most of the gene expression changes that occurred with senescence [11].

As mentioned previously, senescence-associated genes (which are predominantly inflammatory/immune system related) are progressively up-regulated during aging and into senescence, and this up-regulation is universal throughout all 4 types of senescence. Inversely, the inflammatory/immune system pathway is epigenetically abrogated during cellular immortalization. The up-regulation of the inflammatory pathway is most likely due to the expression of the senescence-associated secretory phenotype, whereas the silencing of this pathway during immortalization seems paradoxical. It is unclear why it would be advantageous for an immortal cell to turn off a protective mechanism such as the innate immune system response. It is possible that the cells with a defective interferon response/immune system pathway have a higher miRNA tolerance [59]. Previous studies in the Tainsky lab showed that immortal cells with a dysfunctional interferon response (MDAH087-10) had increased levels of miRNAs in response to DICER expression, whereas immortal cells with a functional interferon response (MDAH087-N) had decreased levels of miRNAs in response to DICER expression. miRNAs typically serve as negative regulators of gene expression, and therefore cells without a functional interferon response and consequently higher levels of miRNAs would have high levels of gene repression. This could cause repression of senescence-associated genes or cell cycle inhibitory genes that would hinder the growth of a cell, and therefore represent an advantage of having an abrogated interferon response.

Similarly, knockdown of DICER in endometrial cancer cell lines causes increased growth and migration, concurrent with decrease in miRNA levels [92]. In conjunction with lower total levels of miRNA expression, there was an overall increase in interferon-stimulated genes, which is consistent with the Tainsky study [92]. This suggests that silencing of the interferon pathway during immortalization confers a higher miRNA tolerance than cells with a normal interferon response.

4.6 Senescence as an Approach to Cancer Therapy

Cancer cells have bypassed the senescent response in order to achieve unrestricted growth. Therefore, re-activation of the senescent response is a logical approach to cancer treatment. However, the ability to exploit senescence during cancer treatment will be complicated and require a deeper understanding of the pathways involved. For instance, it may be possible to inhibit senescence in an effort to increase the amount of apoptotic cell death during cancer treatment, such as treatment with adriamycin. It has been shown that there are a portion of tumor cells that undergo senescence and not apoptosis in response to chemotherapeutic agents [52], and this could be one reason for tumor dormancy. “Irreversibly” senescent cells may be a misnomer and these cells may be able to re-enter the cell cycle if additional mutations or changes in gene expression are acquired. Preliminary experiments have shown that cells treated with H_2O_2 undergo senescence, but are able to begin proliferating again after a short cell cycle arrest (data not shown). In contrast, cells that were treated with adriamycin entered senescence and slowly began dying, presumably going through apoptosis but were not able to start proliferating again (data not shown). Therefore, senescence may not be as irreversible as it seems. RNA-seq data from this study showed that in genes differentially expressed in adriamycin-induced senescence compared to immortalization, 150 of these genes could be grouped into the

apoptosis pathway gene set as defined by Genomatix Genome Analyzer (150 out of 1314 known apoptosis genes, with a p-value of 4.24^{-6}), indicating that the cell may have the option of apoptosis or senescence. H_2O_2-induced senescence had 32 out of 1314 known apoptosis genes, with a p-value of 2.72^{-5}, again indicating induced senescence can be an alternative to apoptosis depending on the circumstance.

Conversely, it may be beneficial to favor senescence as an outcome for chemotherapy rather than try to inhibit it, assuming that senescence is truly "irreversible". However, the senescence-associated secretory phenotype will need to be considered in this complex situation because the secreted factors can have a complicated impact on surrounding cells, both promoting bystander senescence of normal cells and bystander transformation of pre-malignant cells, as well as potentially increasing the overall level of inflammation. Perhaps favoring senescence as an outcome will be beneficial if the senescence-associated secretory phenotype is inhibited simultaneously in an effort to decrease the effects of the secreted factors. For example, it has been shown that inhibition of IL1α can decrease the levels of factors that are secreted as a result of senescence [68]. It may also be possible to induce senescence as a cancer therapy, and concurrently stimulate the immune system to clear the senescent cells before they are able to secrete inflammatory factors that may be detrimental to the surrounding microenvironment by causing bystander senescence or bystander transformation.

4.7 Summary

Comparison of 4 types of cellular senescence showed a common involvement of the inflammatory/immune system pathway during senescence. Further analysis showed heterogeneous involvement of certain inflammatory genes, such as IRF5 and IRF7,

indicating that while certain pathways are universal during senescence, the genes involved from those pathways can vary among different types of senescence. I specifically confirmed the up-regulation of the IL1 pathway in all types of senescence, and in multiple cell lines. It is probable that this pathway plays a causative role in the senescence-associated secretory phenotype, which was also present in all 4 types of senescence, though to varying degrees. This RNA-seq study utilized a cell model which allowed a comparison between multiple types of senescence within the same genetic background, and demonstrated a universal role for the inflammatory pathway in multiple types of senescence, as well as a progressive up-regulation of senescence-associated inflammatory genes as a cell ages.

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ABSTRACT

PATHWAY PROFILING OF REPLICATIVE AND INDUCED SENESCENCE

By

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August 2014

Advisor: Dr. Michael A. Tainsky

Major: Cancer Biology

Degree: Doctor of Philosophy

Senescence is a permanent withdrawal from cell cycle that occurs naturally in cells in response to the shortening of telomeres. This natural "clock" serves to limit the number of cell divisions and therefore protects the cell from potentially carcinogenic mutations. However, senescence also occurs in response to external stresses to the cell, which is known as induced senescence. This study compares the mechanisms of natural senescence, a response to the shortening of telomeres during replication, with induced senescence by using various drugs to induce senescence: 5-aza-2-deoxycytidine (a demethylating agent), Adriamycin (a chemotherapeutic drug), and H_2O_2 (an agent causing oxidative stress).

MDAH041 cells, which are fibroblasts isolated from a patient with Li Fraumeni Syndrome, have heterozygous alleles of p53 and can therefore undergo natural senescence with serial cell culture or at a low frequency spontaneously immortalize once the wildtype copy of p53 is lost. Therefore, this cell model provides naturally senescent cells as well as immortal cells which can be treated with the aforementioned drugs resulting in induced senescence. Using these conditions, gene expression profiling was performed. Gene expression analysis revealed 48 genes differentially expressed in all 4 senescence types

compared to the immortal control. Pathway analysis of these 48 genes from all types of cellular senescence revealed several pathways, each of which are involved in innate immunity, showing for the first time a common gene expression profile among different types of senescence, as well as a central role for the IFN pathway in both natural and induced senescence. Specifically, I have focused on the IL1 pathway which is up-regulated in all types of senescence compared to immortal proliferating cells and will be the basis for additional mechanistic studies.

AUTOBIOGRAPHICAL STATEMENT

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EDUCATION:

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Wayne State University, School of Medicine

Graduate Study

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